14-3-3 isoforms specificity in barley

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geboren te Heemskerk

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prof.dr. J.N.M. Mol

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voor mezelf

Contents

Chapter 1

General introduction

A modified version of this chapter has been submitted for publication
in collaboration with Albertus H. de Boer

14-3-3 history

For an organism to survive under varying environmental conditions it is crucial to exert control over cellular processes like the cell cycle, ion transport, gene expression and enzyme activity. Regulation of the activity and function of proteins, and thereby cellular processes, is an area of great interest in biochemistry. Understanding the mechanism by which organisms regulate protein activity and function potentially allows manipulation of these control mechanisms in for example treating diseases/infections or increasing yield. In recent years, the family of 14-3-3 proteins has emerged as an important regulator of the function and activity of a still rapidly increasing number of proteins.

In 1967, Moore and Perez initially identified 14-3-3 proteins as acidic, abundant proteins in a three-dimensional screen for brain specific proteins. In fact, the name "14-3-3" refers to the fractions in which the proteins were located following DEAE-cellulose chromatography and starch-gel electrophoresis (Moore and Perez, 1967). It took until 1982 to discover that 14-3-3 proteins are not restricted to brain tissue but present in all human tissues, though not in the high concentrations that are found in brain tissue (Boston et al., 1982). Further, 14-3-3 proteins were reported to consist of a family of multiple 14-3-3 isoforms (Boston et al., 1982). Separation of bovine brain 14-3-3 proteins by reverse-phase HPLC showed the presence of at least seven 14-3-3 isoforms, which were denoted with the greek α to η in the eluting order (Ichimura et al., 1988). Another 10 years later, 14-3-3 proteins were discovered in several plant species (Brandt et al., 1992; De Vetten et al., 1992; Hirsch et al., 1992; Lu et al., 1992) and yeast (Van Heusden et al., 1992). Nowadays, 14-3-3 proteins are thought to be ubiquitously expressed in all eukaryotic tissues.

Genomic sequencing projects show that the number of 14-3-3 isoforms present in an organism range from two, in *Saccharomyces cerevisiae*, to a record of potentially fifteen, in *Arabidopsis thaliana* (Rosenquist et al., 2000; Rosenquist et al., 2001). Twelve of these fifteen 14-3-3 coding genes in *Arabidopsis* were shown to be expressed (Rosenquist et al., 2001). It is unclear whether the other three genes represent pseudogenes or are expressed

in yet untested environmental conditions/tissues. In mammals, seven different isoforms were eventually identified, named 14-3-3β, γ, ε, ζ, η, σ and τ. Two of the initially identified 14-3-3 isoforms, 14-3-3α and δ (Ichimura et al., 1988), turned out to be post-translational modifications of 14-3-3β and ζ, respectively (Aitken et al., 1995). In plants, there seems to be a divergence in the number of isoforms between dicotyledons and monocotyledons. Where twelve functional 14-3-3 isoforms were found in dicots like *Arabidopsis* (Rosenquist et al., 2001) and tomato (Roberts, 2003), only eight 14-3-3 isoforms were identified by analysing the rice genome (Jin et al., 2005). In barley, which was among the first plants in which 14-3-3 proteins were discovered (Brandt et al., 1992), only three 14-3-3 isoforms (A, B and C) are described in literature thus far (Brandt et al., 1992; Testerink et al., 1999).

Structural properties

The amino acid sequence of the 14-3-3 proteins is very well conserved, both between the different isoforms within an organism as well as between evolutionary divergent species like yeast, human and barley. Crystal structures of the human 14-3-3τ (Xiao et al., 1995), 14-3-3ζ (Liu et al., 1995) and 14-3-3σ (Wilker et al., 2005) and the tobacco 14-3-3c (Wurtele et al., 2003) have been elucidated and the structures of these isoforms were essentially identical.

The 14-3-3 proteins function as homo- and heterodimers (Jones et al., 1995; Chaudhri et al., 2003), forming a clamp-like structure (Fig. 1). Each individual monomer consists of nine α-helices (A to I), arranged in a “U” shape. Target proteins were found to interact, in the central cavity of the “U” shape which is also called the amphipathic groove, with surface exposed residues of helices C, E, G and I (Wang et al., 1998; Rittinger et al., 1999; Wilker et al., 2005). Dimer formation is accomplished by the first four helices from the N-terminal part of the protein and involves interactions from residues in helices A and B of one monomer with residues in helices C and D of the other monomer (Liu et al., 1995; Xiao et al., 1995; Wu et al., 1997; Wilker et al., 2005). These characteristics are conserved in most 14-3-3 isoforms, enabling them to form most combinations of homo-

and heterodimers. An exception is reported for 14-3-3σ, in which a few residue substitutions in the N-terminal part cause a strong favour for homodimerization (Wilker et al., 2005).

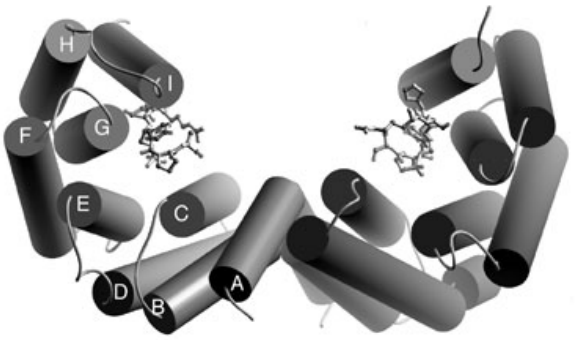

Figure 1. Cartoon representing the three-dimensional crystal structure of a 14-3-3 dimer. Each individual monomer consists of nine α-helices (A to I). Peptides representing the phosphorylated target motif are bound in the central cavity of the negatively charged grooves. (figure adapted from http://web.mit.edu/biology/www/facultyareas/facresearch/yaffe.shtml)

Structural variations between the 14-3-3 isoforms appear to be located mostly in the more divergent C-terminal region of the proteins. These variations in this C-terminal region might play an important role in target interaction/recognition, as indicated by the change in affinity for target proteins upon progressive removal of the C-terminal tail (Truong et al., 2002; Shen et al., 2003). In support of this, three unique residues (Met-202, Asp-204 and His-206) between helix H and I were recently shown to be responsible for ligand discrimination by 14-3-3σ (Wilker et al., 2005). Further, some 14-3-3 isoforms contain a distinct 14-3-3 binding motif in their C-terminal tail. This correlates with the observation that the C-terminal tail might function as an autoinhibitor within the ligand binding groove (Kubala et al., 2004).

The C-terminal region is also subject to regulational modifications. For example, at least some plant 14-3-3 isoforms contain a so-called EF hand-like structure between helix H and I (Lu et al., 1994). This EF hand-like structure, which is missing in animal 14-3-3 proteins, was shown to bind divalent cations and consequently induce a conformational change in the C-terminal tail (Lu et al., 1994). Mutating amino acid residues in this EF hand-like structure affected the cation dependence of 14-3-3 in regulating the target nitrate reductase (NR) (Athwal and Huber, 2002). Another example of regulational modification

of 14-3-3 proteins is by phosphorylation. Several phosphorylation sites have been reported in, mainly mammalian, 14-3-3 isoforms (Aitken, 2002; Giacometti et al., 2004). In most cases the consequences of phosphorylation on protein structure/function are not clear yet. Some phosphorylations near the N-terminus are suggested to regulate dimer formation (Aitken, 2002; Powell et al., 2002; Powell et al., 2003). Further, several reports show inhibition of 14-3-3 binding to their target protein upon phosphorylation of the 14-3-3 (Dubois et al., 1997; Fu et al., 2000; Van der Hoeven et al., 2000; Aitken, 2002; Tzivion and Avruch, 2002; Yoshida et al., 2005). The best-studied examples of the effect of 14-3-3 phosphorylation are found in mammalian signal transduction pathways. For example, *in vivo* phosphorylation of 14-3-3ζ, on the C-terminal Thr-233, negatively regulates its interaction with c-Raf (Dubois et al., 1997). The interaction between 14-3-3 and c-Raf is necessary to keep c-Raf in an inactive but activation competent confirmation. The c-Raf protein is a component of the MAPK pathway that is involved in processes like cell differentiation and apoptosis. For plant 14-3-3 proteins, little is known about the phosphorylation status and the possible effects of phosphorylation on the activity of the 14-3-3 proteins, although an early report already mentioned phosphorylation of 14-3-3 proteins at Ser residues (Lu et al., 1994). Thus far, the only known effect has been described for Tyr-137 phosphorylation of maize 14-3-3 isoform GF14-6, which lowered the affinity of GF14-6 for the target plasma membrane H^+-ATPase (Giacometti et al., 2004). For a better understanding of the function and target interaction/recognition of 14-3-3 proteins, the regulation of 14-3-3 activity should and will be explored in more detail in the future.

Functions and targets of 14-3-3 proteins

The regulatory functions ascribed to 14-3-3 proteins are diverse; they include controlling metabolic enzymes (Bachmann et al., 1996b; Toroser et al., 1998; Cotelle et al., 2000; Huber et al., 2002), ion transport activity (Bunney et al., 2001; Van den Wijngaard et al., 2001; De Boer, 2002; Plant et al., 2005; Sinnige et al., 2005b; Van den Wijngaard et al., 2005), protein kinases (Aitken et al., 1992; Irie et al., 1994; Reuther et al., 1994; Camoni et al., 1998; Feng et al., 2005; Otterhag et al., 2005), gene transcription (De Vetten et al.,

1992; Lu et al., 1992; Schultz et al., 1998; Pan et al., 1999), protein assembly (O'Kelly et al., 2002; Rajan et al., 2002) and targeting of proteins (Jarvis and Soll, 2002; Rajan et al., 2002; Van Hemert et al., 2004; Paul et al., 2005). Although the exact function of 14-3-3 proteins is not completely understood, the mechanism by which 14-3-3 proteins accomplish these diverse functions is basically the same. With few exceptions, the amphipathic groove of 14-3-3 proteins bind to distinct phosphorylated motifs, R/KxxS^{P}/T^{P}xP (mode-1) and R/KxxxS^{P}/T^{P}xP (mode-2) in which S^{P}/T^{P} indicates a phosphorylated serine/threonine, in their target proteins (Muslin et al., 1996; Yaffe et al., 1997). Interactions of 14-3-3 proteins with their target molecules are initiated by the phosphorylation of these target molecules and can involve, due to the dimeric nature of the 14-3-3 proteins, one or two interactions at the same time. Upon binding of a 14-3-3 protein, the functionality of the target protein(s) is altered. This can be accomplished by either activation/inactivation of the activity of the target, by (prevention of) translocation of the target to a different cellular compartment, or by interaction with other molecules that are in the same complex.

At first sight there seems to be a difference in 14-3-3 target proteins between animals and plants. Most of the well characterised animal 14-3-3 target proteins described in literature are involved in signalling, while in plants most target proteins are enzymes of primary metabolism. However, with the recent large-scale purifications of 14-3-3 target proteins (Milne et al., 2002; Pozuelo Rubio et al., 2004; Benzinger et al., 2005) it became evident that this apparent difference between animals and plants might have been a difference in focus/research interest between these fields.

In animals

The first proteins identified to interact with 14-3-3 proteins were tryptophan 5-monooxygenase and tyrosine 3-monooxygenase (Ichimura et al., 1987), rate-limiting enzymes in indolamine (e.g. serotonin and melatonin) and catecholamine (e.g. dopamine, noradrenaline and adrenaline) biosynthesis, of which the activity was stimulated upon 14-3-3 binding (Ichimura et al., 1987). The list of known 14-3-3 targets nowadays contains

hundreds of proteins and is growing rapidly. Not surprisingly, some of the best-studied examples of regulation by 14-3-3 proteins are related to human diseases.

Signal transduction pathways with a potential for oncogenic transformations are extensively studied and 14-3-3 proteins are reported to interact with many of the proteins involved (Hermeking, 2003; Wilker and Yaffe, 2004). The most intriguing is probably the previously mentioned kinase c-Raf, as it contains at least four 14-3-3 binding sites (Morrison et al., 1993; Muslin et al., 1996; Clark et al., 1997; Dumaz and Marais, 2003) of which some are inhibitory (Roy et al., 1998; Light et al., 2002) and some are stimulatory (Thorson et al., 1998) upon 14-3-3 binding. To further complicate the picture, proteins directly upstream (Ras and protein kinase C) and downstream (mitogen activated protein kinase kinase MEK) of c-Raf also interact with 14-3-3 proteins, which might have a scaffolding function. A more direct involvement in aetiology of cancer is reported for 14-3-3σ, of which the down-regulation is observed in many tumor types and may be an early event in oncogenic transformation (Hermeking, 2003; Wilker and Yaffe, 2004; Lodygin and Hermeking, 2005). Although the precise mechanism remains unclear, 14-3-3σ appears to be an essential component to accomplish G2/M arrest upon DNA damage. This is possibly accomplished through interaction with the CDC2-cyclin-B1 complex, shown to coimmunoprecipitate with a 14-3-3σ specific antibody (Chan et al., 1999), and sequestering this complex in the cytosol. However, the partners in this complex were not identified in a recent affinity purification with 14-3-3σ, identifying 117 potential target proteins (Benzinger et al., 2005).

The possible involvement of 14-3-3 proteins in various neurological disorders has recently attracted a lot of interest (Berg et al., 2003; Teunissen et al., 2005). 14-3-3 proteins are detected in the cerebrospinal fluid of patients with for example, severe forms of, multiple sclerosis (Teunissen et al., 2005). For the diagnosis of Creutzfeldt-Jakob disease, detection of 14-3-3 proteins in the cerebrospinal fluid is even a standard diagnostic criterium (Hsich et al., 1996; Zerr et al., 1998; Beaudry et al., 1999). Further, increased levels of some 14-3-3 isoforms were shown in several brain regions in patients suffering from Alzheimer's disease (Layfield et al., 1996; Fountoulakis et al., 1999). Various

binding partners of 14-3-3 are involved in apoptosis and/or vulnerability for neurodegradation (e.g. Bad and Fkhrl1) (Berg et al., 2003). However, so far, it is unclear what role 14-3-3 proteins play in these diseases.

The 14-3-3 proteins also serve an interesting function in the development of embryos. One of the partition-defective PAR proteins, which are responsible for the establishment of anterior-posterior polarity and gastrulation in the embryonic stage of *C. elegans* (Morton et al., 2002), *Drosophila* (Benton et al., 2002) and *Xenopus* (Kusakabe and Nishida, 2004), is identical to a 14-3-3 protein. A similar function of 14-3-3 was shown in establishing and maintaining epithelial polarity in mammalian cells (Suzuki et al., 2004). Intriguingly, 14-3-3 proteins also function in left-right patterning during embryogenesis (Bunney et al., 2003). 14-3-3e is asymmetrically localized in the earliest stages of *Xenopus* development and disruption of this asymmetrical localization leads to severe heterotaxia in the developing embryos (Bunney et al., 2003).

In plants

As mentioned above, most of the identified and characterized 14-3-3 targets in plants are enzymes involved in the primary metabolism (Huber et al., 2002; Comparot et al., 2003). In line with this, manipulation of the 14-3-3 content in potato, using silencing and overexpression techniques, resulted in an altered lipid, sugar and starch content (Prescha et al., 2001; Szopa et al., 2001). Further, the amino acid and mineral composition of these plants were reported to be slightly changed (Swiedrych et al., 2002). An example of the potential control 14-3-3 proteins exert on metabolic pathways is found in the carbohydrate metabolism (Fig. 2). Sucrose is a major product of photosynthesis in plants and used as a long-distance transport compound. The conversion of fructose-6-phosphate and UDP-glucose to sucrose-6-phosphate is catalysed by sucrose-phosphate synthase (SPS) and, among other regulatory effectors, inhibited by binding of 14-3-3 proteins (Toroser et al., 1998). Besides the synthesis of sucrose, the hydrolysis of sucrose is also, at least partly, under control of 14-3-3 proteins. The degradation of sucrose into hexoses can be catalysed by both sucrose synthase and invertases, of which the cytosolic neutral invertase is shown to be inhibited by 14-3-3 proteins (Chapter 4). Finally, 14-3-3 proteins were found to

interact with several enzymes in pathways that, directly (trehalose-6-phosphate synthase and fructose-2,6-biphosphatase) or indirectly (starch synthase III and glyceraldehyde-3-phosphate dehydrogenase), use the hexose phosphate pool as a substrate (Moorhead et al., 1999; Sehnke et al., 2001; Bustos and Iglesias, 2003; Kulma et al., 2004; Zuk et al., 2005). Possibly, the 14-3-3 proteins are in this way involved in the distribution of the available hexoses over the different pathways.

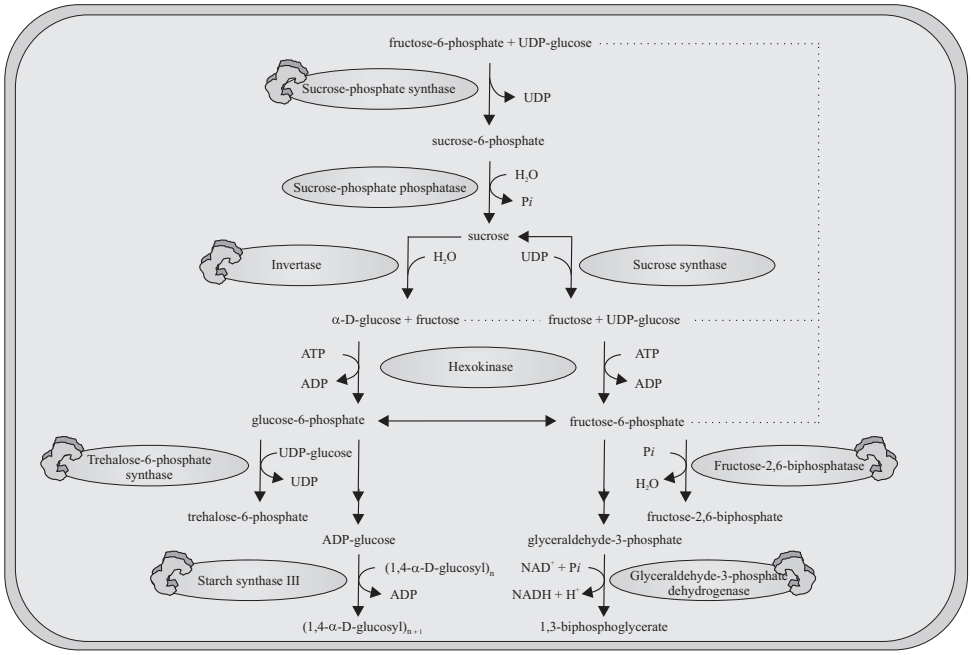

Figure 2. Role of 14-3-3 proteins in part of the carbohydrate metabolism of plants. By binding to several proteins in the carbohydrate metabolism, 14-3-3 proteins seem to control the availability and distribution of hexoses. Both the synthesis and the hydrolysis of sucrose are, at least partly, inhibited upon binding of 14-3-3 proteins to sucrose-phosphate synthase and invertase, respectively. Further, several enzymes that, directly or indirectly, consume hexoses as a substrate are regulated by interaction with 14-3-3 proteins. The indirect consumers, stach synthase III and glyceraldehyde-3-phosphate dehydrogenase, are both inhibited in their activity by 14-3-3 interaction. The effect of 14-3-3 binding on the activity of trehalose-6-phosphate synthase and fructose-2,6-biphosphatase is yet unclear.

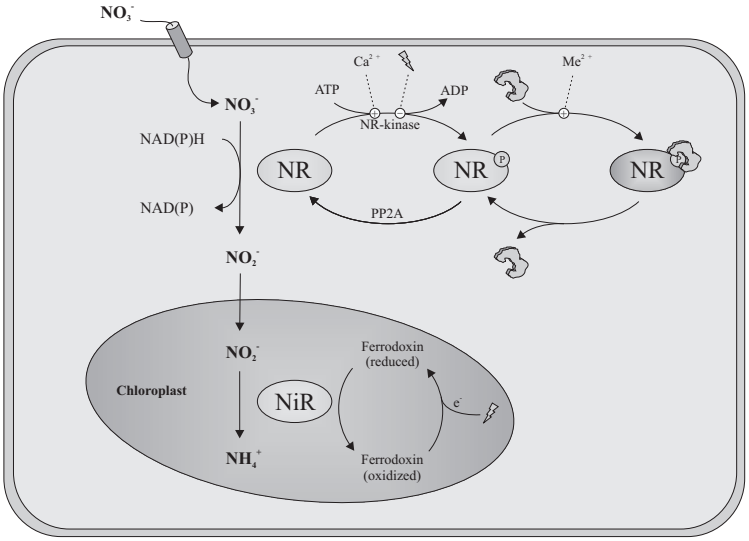

Figure 3. Role of 14-3-3 proteins in the regulation of nitrate reductase (NR) activity. Nitrate is taken up by the plant cell though nitrate transporters and subsequently reduced to nitrite by NR. In the chloroplast, nitrite reductase (NiR) further reduces nitrite to ammonium at the cost of oxidising ferrodoxin. The reduction of ferrodoxin requires an electron, which is produced by photosynthetic activity. Upon darkness, the photosynthetic activity stops and NiR is unable to reduce nitrite to ammonium. To prevent accumulation of the toxic nitrite, NR is rapidly phosphorylated by NR-kinase. The activity of this kinase is inhibited by light and stimulated by cytosolic Ca^{2+}. Subsequent binding of 14-3-3 proteins to the phosphorylated NR completely inhibits the activity of NR.

The best-studied enzyme in plants concerning 14-3-3 regulation is NR (Fig. 3) (Huber et al., 1996; Kaiser and Huber, 2001; Huber et al., 2002; Kaiser et al., 2002; Comparot et al., 2003). Nitrate, the main nitrogen source for plants, is reduced to nitrite by NR. Nitrite is further reduced to ammonium by nitrite reductase (NiR) and ammonium is subsequently used in for example the amino acid biosynthesis. For the reduction of nitrite, NiR depends on electrons produced by photosynthetic activity that are transferred through ferrodoxin. To prevent accumulation of the toxic nitrite in times of darkness, NR is phosphorylated at Ser-543 (in spinach) by a Ca^{2+} stimulated and light inhibited NR-kinase (Douglas et al., 1995; Mackintosh et al., 1995; Lillo et al., 2004). Subsequent binding of 14-3-3 to the phosphorylared NR inactivates the activity of NR (Bachmann et al., 1996b; Moorhead et al., 1996). The interaction between NR and 14-3-3 proteins is, at pH 7.5, stimulated by the

presence of certain polyamines and/or millimolar concentrations of divalent cations (Bachmann et al., 1996b; Athwal et al., 1998; Athwal and Huber, 2002). This stimulation seems to be mediated through the previously discussed EF-hand like structure in loop 8 of plant 14-3-3 proteins (Lu et al., 1994; Athwal and Huber, 2002). Interestingly, binding of 14-3-3 to NR was also suggested to induce proteolysis of NR (Weiner and Kaiser, 1999). Later work, however, indicated that 14-3-3 proteins are released from target proteins, including NR and SPS, prior to proteolysis, which is initiated by reducing sucrose levels (sugar starvation) (Cotelle et al., 2000; Kaiser and Huber, 2001).

By studying the effect of the phytotoxin fusicoccin (FC), 14-3-3 proteins emerged as regulators of plant ion homeostasis (Bunney et al., 2002; De Boer, 2002). FC was found to stimulate the atypical binding of 14-3-3 to the autoinhibiting C-terminal end of the plasma membrane H^+-ATPase, thereby increasing the activity of the pump (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). More recently, 14-3-3 proteins were also shown to inhibit the mitochondrial and chloroplast F_oF_1 ATP synthase (Bunney et al., 2001), which are evolutionary unrelated to the plasma membrane H^+-ATPase. Beside the effect on proton transport, 14-3-3 proteins were shown, using electrophysiological experiments, to affect several potassium channels in the plasma (Saalbach et al., 1997; Booij et al., 1999; Van den Wijngaard et al., 2005) and vacuolar membranes (Van den Wijngaard et al., 2001; De Boer, 2002; Sinnige et al., 2005b). Interestingly, FC is also reported to stimulate K^+ uptake (Marre, 1979). Possibly this is accomplished through a similar mechanism, involving a stimulation of 14-3-3 binding and subsequent inhibition of plasma membrane outward K^+ currents, as found for the plasma membrane H^+-ATPase (Blatt and Clint, 1989; Van den Wijngaard et al., 2005). However, the mechanism through which 14-3-3 proteins and/or FC affect the magnitude of K^+ currents in plants is still poorly understood. In fact, until recently (Sinnige et al., 2005b) there was no report that described the molecular basis of interaction between 14-3-3 proteins and an ion channel.

Although hundreds of 14-3-3 target proteins have been described, the potential number of 14-3-3 targets is much larger. As mentioned before, with few exceptions, 14-3-3 proteins interact with distinct motifs in their target proteins. This knowledge was used by Sehnke and colleagues (2002a) to screen the predicted protein products of the sequenced

Arabidopsis genome for potential 14-3-3 target proteins. More than 10 000 proteins, representing approximately half the proteins in *Arabidopsis*, contained at least one mode-1 and/or mode-2 motif. Obviously, this does not imply that all these proteins are definite targets of 14-3-3 proteins, as the motif needs to be accessible and, in most cases, phosphorylated for 14-3-3 interaction. However, the current developments in proteomics (Milne et al., 2002; Pozuelo Rubio et al., 2004; Benzinger et al., 2005; Satoh et al., 2005) do indicate that the number of 14-3-3 target proteins is very large indeed and comparable large scale identification of plant 14-3-3 interacting proteins should provide a better insight in the functions 14-3-3 proteins exert in plants.

Isoform specificity

The abundance of regulatory functions and (potential) targets of 14-3-3 proteins, begs the question of how 14-3-3 proteins discriminate between their diverse tasks/target proteins. Sure, a significant contribution to this discrimination will be in the hands of protein kinases, which control the phosphorylation status of 14-3-3 target proteins. Further, the presence of multiple 14-3-3 isoforms could contribute to this discrimination, assuming that each isoform is specialised in a subset of target proteins. Whether 14-3-3 molecules discriminate between target proteins or are functionally redundant is currently one of the major issues in 14-3-3 biology.

Analysing the structural properties of 14-3-3 isoforms provides little evidence for isoform specific interactions, since residues in the amphipathic groove of 14-3-3 isoforms that are involved in ligand binding are highly conserved (Zhang et al., 1997; Petosa et al., 1998; Wang et al., 1998). Furthermore, in peptide screens, different 14-3-3 isoforms seem to prefer interaction with similar target motifs (mode-1 and mode-2) and bind to these motifs with similar affinities (Muslin et al., 1996; Yaffe et al., 1997). Moreover, several *Arabidopsis* 14-3-3 isoforms were able to complement the lethal deletion of the corresponding yeast genes (Van Heusden et al., 1996; Kuromori and Yamamoto, 2000). Based on these indications for functional redundancy, the existence of multiple 14-3-3 isoforms was suggested to function in providing each cell/cellular compartment with the desired quantity of 14-3-3 proteins (Palmgren et al., 1998). Thus, 14-3-3 isoforms are

redundant in their ability to act on target proteins and specificity reflects expression patterns and availability of 14-3-3 proteins (Roberts, 2000; Roberts and de Bruxelles, 2002; Zuk et al., 2005). In support of this theory, differential expression of 14-3-3 proteins is indeed observed in specific cell types (Daugherty et al., 1996; Testerink et al., 1999; Roberts and de Bruxelles, 2002; Sehnke et al., 2002b; Maraschin et al., 2003b) and throughout development (Testerink et al., 1999; Maraschin et al., 2003a). Further, some 14-3-3 isoforms were found to be subcellular localised (Martin et al., 1994; Sehnke et al., 2000; Bunney et al., 2001; Van Hemert et al., 2004; Paul et al., 2005), affecting the availability of these 14-3-3 isoforms.

On the other hand, evidence is accumulating for isoform specific functional differences of 14-3-3 proteins. By analysing several 14-3-3 isoforms in the same experimental system, Bachmann et al. (1996a) found significant differences in the ability of 14-3-3 isoforms to inhibit NR. These functional differences correlate with the affinity of these isoforms towards a synthetic peptide, which represented the 14-3-3 binding domain of NR, indicating that the observed functional differences are a direct consequence of the ability of the 14-3-3 isoforms to bind NR (Bachmann et al., 1996a). Similar functional differences between 14-3-3 isoforms are observed in the regulation of the plasma membrane H^+-ATPase (Rosenquist et al., 2000; Emi et al., 2001; Alsterfjord et al., 2004), the slow-activating vacuolar (SV) channel (Sinnige et al., 2005b) and sucrose-phosphate synthase (Bornke, 2005). Functional isoform specificity *in vivo*, might be a consequence of small affinity differences between different 14-3-3 isoforms. Therefore, complementation experiments prove little redundancy as (over-)expression of 14-3-3 in a heterologous system may override (subtle) differences in affinity. Interestingly, the observed subcellular localisation of 14-3-3 isoforms was recently shown to be mainly target driven and is therefore rather an indication for functional isoform specificity (Paul et al., 2005). Further, differential expression of 14-3-3 proteins can hardly be seen as evidence against functional isoform specificity, although the availability should obviously be taken into account in assigning functions to the different 14-3-3 isoforms. However, the molecular basis for isoform specificity has long been missing.

Recently, natural varying residues in loop 8 of 14-3-3 proteins were shown to function in ligand discrimination (Sinnige et al., 2005a; Wilker et al., 2005). Variations in this region might alter the position of helix 9 and the C-terminal tail of 14-3-3 proteins. Possibly, this affects the accessibility of the amphipathic groove, as helix 9 is involved in the formation of this ligand binding groove (Petosa et al., 1998) and the C-terminal tail functions as an auto-inhibitor (Truong et al., 2002; Shen et al., 2003) by occupying the amphipathic groove (Kubala et al., 2004; Silhan et al., 2004). Alternatively, the residues in loop 8 could function directly in ligand recognition (Wilker et al., 2005) as direct contact has been observed between residues in loop 8 and part of a co-crystallized ligand (Obsil et al., 2001).

The observed functional differences between 14-3-3 isoforms in regulating a variety of target proteins in combination with the identification of natural varying residues that function in ligand discrimination strongly indicates functional isoform specificity in 14-3-3 regulation. Additional mutagenesis of natural variations between 14-3-3 isoforms and analysis of the structural consequences are necessary to fully understand ligand discrimination.

Outline thesis

Analysis of the capacity of 14-3-3 isoforms to regulate different target proteins in combination with their availability should provide a better insight in the suggested functional differences of 14-3-3 isoforms and possibly identify the molecular basis for isoforms specificity in 14-3-3 binding. This thesis describes our quest to obtain insight in the regulatory functions of 14-3-3 proteins in barley.

The initial focus of this work was on the regulation of K^+ channels by 14-3-3 proteins. The currents of several K^+ conducting channels were shown, using electrophysiological techniques, to be affected by addition of 14-3-3 (Saalbach et al., 1997; Booij et al., 1999; Van den Wijngaard et al., 2001; De Boer, 2002). However, the molecular mechanism behind this effect remained unclear, partly due to the lack of knowledge about which proteins/genes were responsible for the observed K^+ currents. Our efforts to isolate genes

from barley that code for K^+ transporters resulted, amongst others, in the isolation of *HvKCO1*, orthologue of the previously described *Arabidopsis AtKCO1* (Czempinski et al., 1997). As described in Chapter 2, a distinct 14-3-3 binding motif is conserved in the N-terminal region of all KCO1 orthologues from different plant species. While attempting to identify the corresponding K^+ current, the protein was reported to be a component of the SV channel (Schönknecht et al., 2002), which is indeed regulated by 14-3-3 (Van den Wijngaard et al., 2001). Upon studying the ability of the 14-3-3 binding motif from HvKCO1 to bind the thus far described barley 14-3-3 isoforms (A, B and C), 14-3-3A was found to interact with the highest affinity, followed by the B- and C- isoforms. Interestingly, the complete opposite order was found when studying the ability of the 14-3-3 isoforms to reduce the barley SV current (Chapter 2). This inconsistency is in agreement with the very recent observation that the SV current is not mediated through KCO1, but through TPC1 (Bihler et al., 2005; Peiter et al., 2005).

The apparent differences in affinity for barley 14-3-3 isoforms were further explored using one of the model systems for 14-3-3 action in plants, the inhibition of NR (Chapter 3). Clear differences in both functional capability as in availability of the different 14-3-3 isoforms were observed, leaving 14-3-3B as the only characterised isoform able to regulate NR activity in barley. More importantly, upon comparing the amino acid sequences of the 14-3-3 isoforms, an educated guess led to the identification of (part of) the molecular basis for isoform specificity. A single amino acid variation in loop 8 of the 14-3-3 proteins was found to play an important role in ligand discrimination.

Chapter 4 describes an affinity purification approach to identify (new) 14-3-3 target proteins. Although promising results were obtained upon testing and optimising the method with the purification of NR, the large-scale purification of 14-3-3 target proteins as described for mammalian systems (Pozuelo Rubio et al., 2004; Benzinger et al., 2005) could not yet be accomplished. Still, a neutral invertase was identified and confirmed as a novel 14-3-3 target that functions in the carbohydrate metabolism. Further, in the light of ligand discrimination between the 14-3-3 isoforms, parallel affinity purifications were performed with the three different isoforms as bait.

Besides the functional capability of 14-3-3 isoforms to interact with target proteins, the availability of 14-3-3 isoforms was investigated. In Chapter 5 we describe the identification of two novel 14-3-3 isoforms (D and E) in barley. The relative expression levels of these five 14-3-3 isoforms were determined in several barley tissues during different stages of development. Because of the possible involvement of 14-3-3 proteins in abscisic acid (ABA) signal transduction (Van den Wijngaard et al., 2005), the hormone that plays a key role during seed germination, special attention was paid to the expression of the 14-3-3 genes during the earliest hours of development and their response to ABA.

Chapter 2

The barley two-pore K^+ channel HvKCO1 interacts with 14-3-3 proteins in an isoform specific manner

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Summary

Members of the highly conserved family of 14-3-3 proteins play an important role in various cellular processes. Recent studies, using electrophysiological techniques, show that the 14-3-3 proteins also regulate plasma membrane and vacuolar K^+ conducting channels. The molecular mechanism behind the regulatory effect of 14-3-3 proteins on K^+ channels remains to be shown. One vacuolar channel down-regulated by 14-3-3 proteins is the slow-activating vacuolar (SV) channel. In *Arabidopsis*, the protein coded by the *KCO1* gene was recently shown to be present in the vacuolar membrane and identified as a component of the SV channel. These two observations raised the question whether the KCO1 protein does interact with 14-3-3 proteins. Therefore, we isolated the barley *HvKCO1* gene and the encoded protein indeed contains a canonical 14-3-3 interaction motif, which is conserved in all other KCO1 orthologues from other plant species. Using surface plasmon resonance (SPR) we determined in real-time the affinity between the phospho-peptide derived from the putative KCO1 14-3-3 interaction motif and three barley 14-3-3 proteins. The 14-3-3A protein showed the highest affinity, whereas the binding of all three isoforms was dependent on the presence of either Ca^{2+} or Mg^{2+}. Interestingly, the barley SV current was strongly reduced by 14-3-3B and C protein, but not by 14-3-3A. This difference between the SPR and patch-clamp data will be discussed, along with the role for Ca^{2+} in activation of the SV channel by direct interaction and inactivation of the channel by facilitating the binding of 14-3-3 to the channel.

Introduction

First described in 1967 as a brain specific protein (Moore and Perez, 1967), the family of 14-3-3 proteins has evolved into a group of abundant regulatory proteins present in all eukaryotic tissues. These 14-3-3 proteins act as dimers and in most cases bind to distinct phosphorylated motifs, R/KxxS^{p}/T^{p}xP and R/KxxxS^{p}/T^{p}xP in which S^{p} indicates a phosphorylated serine, in their target proteins (Yaffe et al., 1997; Yaffe and Elia, 2001). Furthermore, the presence of divalent cations, like Mg^{2+} and Ca^{2+}, seems to increase the affinity for some of those targets (Athwal et al., 1998; Toroser et al., 1998). The highest number of family members, twelve expressed isoforms, has been found in *Arabidopsis* (Rosenquist et al., 2001). Monocots seem to contain fewer isoforms; three (A, B and C) have been described in barley so far (Brandt et al., 1992; Testerink et al., 1999) although our data suggest that at least five isoforms are present (Sinnige et al., unpublished results). The regulatory functions ascribed to 14-3-3 proteins are diverse; they include controlling metabolic enzymes (Huber et al., 2002), protein kinases (Camoni et al., 1998), chloroplast import (Jarvis and Soll, 2002), transcription factors (Eckardt, 2001), the activity of ion transporters (Bunney et al., 2002) and the correct assembling and targeting of ion channels (Rajan et al., 2002).

In recent years 14-3-3 proteins have emerged as novel regulators of plant ion homeostasis (Bunney et al., 2002; De Boer, 2002). Besides modulating the P-type H^{+}-ATPase (Jahn et al., 1997; Baunsgaard et al., 1998) and the mitochondrial and chloroplast F-type ATP synthases (Bunney et al., 2001), 14-3-3 proteins were shown to affect K^{+} channels in the plasma and vacuolar membranes. Over-expression of Vf14-3-3a or Vf14-3-3b in tobacco enhanced outward K^{+} currents (Saalbach et al., 1997) and addition of recombinant 14-3-3 to the cytosolic side of tomato suspension cells doubled the outward K^{+} currents (Booij et al., 1999) of the plasma membrane. However, in the plasma membrane of barley embryonic root tissue, 14-3-3 proteins reduced outward K^{+} currents and an inward rectifying K^{+} current was found to be dependent on the presence of 14-3-3 (Van den Wijngaard et al., 2005). Further, in the vacuolar membrane, two ion channels respond to the addition of recombinant 14-3-3 protein to the bath solution in a patch-clamp set-up. Whereas the current of the fast-activating vacuolar channel increased four-fold (De Boer,

2002), the Ca^{2+} dependent slow-activating vacuolar (SV) current decreased up to 80% after adding recombinant barley 14-3-3B (Van den Wijngaard et al., 2001). The mechanism, through which 14-3-3 proteins affect the magnitude of the currents, without affecting the channel gating properties, is still poorly understood.

Recently, AtKCO1, a member of the two-pore domain K^+ channel family, was reported to be part of the vacuolar SV channel. A reduced SV current in an *Arabidopsis kco1* knockout plant led to the conclusion that AtKCO1 is a component of the SV channel (Schönknecht et al., 2002). In an earlier report, expression of *AtKCO1* in baculovirus-infected insect cells resulted in Ca^{2+} dependent outward rectifying currents (Czempinski et al., 1997). Localization of the protein in the vacuolar membrane was confirmed using AtKCO1-GFP fusion proteins (Czempinski et al., 2002; Schönknecht et al., 2002). The SV channel has been subject of extensive, mainly electrophysiological, research. It is present in all studied plant species, conducts both K^+ and Ca^{2+} ions (Pottosin et al., 2001) and the activity is strongly dependent on cytosolic Ca^{2+} concentrations (Hedrich and Neher, 1987). Besides the inhibition by barley 14-3-3B protein (Van den Wijngaard et al., 2001), the activity of SV is affected by calmodulin (Bethke and Jones, 1994), the redox state (Carpaneto et al., 1999), phosphorylation (Bethke and Jones, 1997) and cytosolic Mg^{2+} (Pei et al., 1999). A physiological role for the SV channel in Ca^{2+} induced Ca^{2+} release (CICR) was proposed (Ward and Schroeder, 1994), challenged (Pottosin et al., 1997) and is still a matter of debate (Bewell et al., 1999; Sanders et al., 1999; Miedema et al., 2003; Pottosin et al., 2004).

Here we report the isolation and analysis of *HvKCO1,* member of the KCO1 family of K^+ channels. Using a conserved motif in the KCO1 family, we demonstrate that 14-3-3 proteins are able to interact with HvKCO1 in the presence of divalent cations. To assess whether 14-3-3 regulation of the SV channel is mediated through interaction with the KCO1 component, we compare the binding properties of 14-3-3 isoforms to HvKCO1 with the effect of the different 14-3-3 isoforms on the SV current in barley leaf vacuoles.

Results

HvKCO1, an orthologue of AtKCO1

To isolate the barley orthologue of *AtKCO1*, the barley EST database of IPK-Gatersleben was searched using *AtKCO1* as a template. One EST was identified as a member of the KCO family of K^+ channels. Using a combination of RACE technology and genome walking we identified the complete coding sequence from *H. vulgare* cv. Alexis. The amino acid sequence of the gene (*HvKCO1*) contains the characteristic two pore domains, four membrane-spanning helices and two putative EF-hands of the KCO K^+ channel family (not shown). *HvKCO1* expression was found in all tested tissues (Fig. 1a). Like previously reported for *AtKCO1* (Schönknecht et al., 2002), expression of *HvKCO1* is highest in leaves, where expression is approximately five times higher than in roots. The presence of transcript in radicles isolated from embryos 20 hours after imbibition, shows that *HvKCO1* is expressed from the earliest stages of development on. The effect of abscisic acid (ABA) on *HvKCO1* expression in barley radicles was tested in the light of a putative function of SV in CICR (Ward and Schroeder, 1994), where oscillations in cytosolic Ca^{2+} concentrations function as second messenger in ABA signaling (Himmelbach et al., 2003), and the recently reported effect of 14-3-3 proteins and ABA on K^+ channel activity in barley embryonic root (Van den Wijngaard et al., 2005). Upon addition of 10 µM ABA to isolated radicles, radicle growth was inhibited as previously described (Van den Wijngaard et al., 2005) and the expression of *HvKCO1* in radicles increased three-fold (Fig. 1b), suggesting a role for KCO1 in ABA signaling. Phylogenetic analysis of *HvKCO1*, the Arabidopsis KCO family and KCO1 orthologues of rice, potato and eucalyptus, confirm *HvKCO1* as a KCO1 orthologue (Fig. 2).

14-3-3 proteins bind to HvKCO1 in an isoform specific manner

Since AtKCO1 was reported to be a component of the SV channel (Schönknecht et al., 2002) and our group previously showed that the SV current is inhibited by 14-3-3B (Van den Wijngaard et al., 2001), we analysed HvKCO1 for the presence of 14-3-3 binding

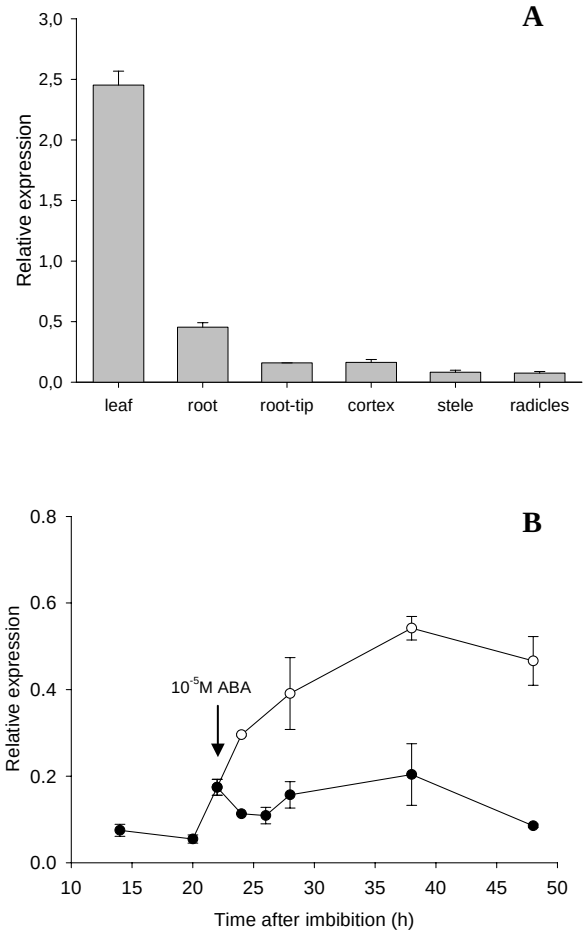

Figure 1. Expression of *HvKCO1* is found in all tested tissues and is induced upon ABA treatment.

(A) Relative expression of *HvKCO1* in different tissues of *Hordeum vulgare*. Radicles were isolated 20 hour after inbibition, leaf and root were harvested from 1-week-old plants; root-tips, cortical and stelar tissue were isolated from 3-week-old secondary roots. Expression was determined using quantitative RT-PCR and normalised to the expression of actin (n = 3; mean ± S.D.).

(B) Effect of 10^{-5}M ABA (○) on the expression of *HvKCO1* in isolated radicles compared with the control situation (●).

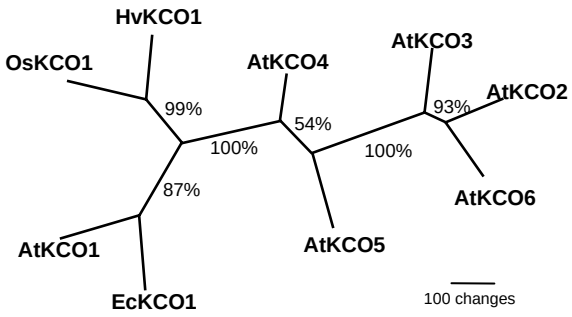

Figure 2. Non-rooted phylogenetic tree with all members of the *Arabidopsis* two-pore K^+ channel family (*AtKCO1-6*) (Maser et al., 2001), *HvKCO1*, *OsKCO1* (GenBank accession no. NM_186155) and *EcKCO1* (GenBank accession no. AF175507) shows that the isolated barley gene is a KCO1 orthologue. Alignment of cDNA's was produced using AlignX (Vector NTI) and the plot produced by Treeview. Values indicate the times (percentage) that branch was found using bootstrap analysis (n = 1000).

```
                  KR
HvKCO1     27  GAKRFRRSRSAPRSE  41
AtKCO1     33  RKRRLRRSRSAPRGD  47
OsKCO1     23  AARRFRRCRTAPSSE  37
StKCO1α    24  GRKRFRRSKSTPVGE  38
StKCO1β    24  RRRRLRRLKSAPMPE  38
EcKCO1     26  KRNRLRRCKSAPLAE  40
                   14-3-3
```

Figure 3. Amino acid alignment of the putative 14-3-3 binding domain in KCO1 proteins from barley, *Arabidopsis*, rice, potato (Czempinski et al., 2002) and eucalyptus. The canonical binding motif (RxxS/TxP) is shown in grey bars (■) and is conserved in all KCO1 proteins of the different plant species. The previously described KR rich region and the 14-3-3 binding sequence is indicated. The alignment of amino acids was produced using AlignX in Vector NTI.

motifs. Indeed, the N-terminus of the HvKCO1, and its orthologues, contain a distinct 14-3-3 binding motif, RRxxS/TxP, similar to the canonical motifs $RxxS^{p}/T^{p}xP$ and $RxxxS^{p}/T^{p}xP$. This motif is located in the previously described KR-rich sequence (Czempinski et al., 1997; Czempinski et al., 2002), it is conserved in all KCO1 orthologues from different plant species (Fig. 3), but it is not present in the other members of the *Arabidopsis* KCO family.

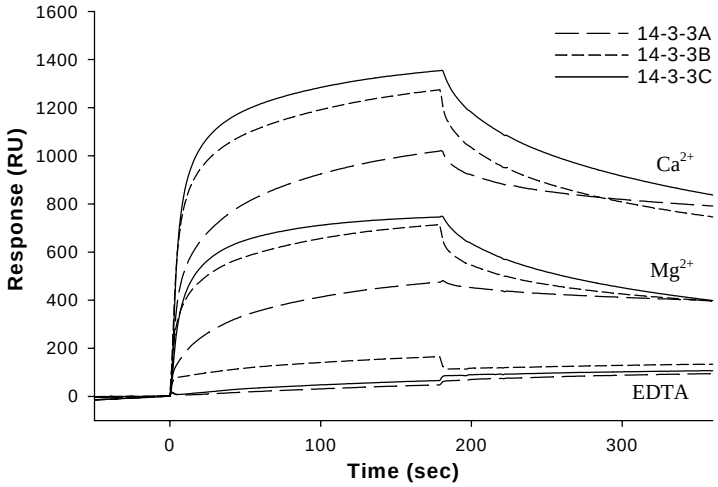

Figure 4. Superimposed surface plasmon resonance sensorgrams showing binding of 14-3-3 isoforms A (–––), B (----) and C (——) to phosphorylated HvKCO1 peptide. Binding was tested in the presence of 5 mM Ca^{2+}, Mg^{2+} or EDTA. Each curve shows three phases: the first phase represents equilibration with running buffer, the second phase shows binding of the 14-3-3 protein to the chip and the third phase shows dissociation of 14-3-3 protein when only running buffer is passed over the chip. Binding of all three isoforms is clearly dependent upon the presence of divalent cations and note that dissociation of 14-3-3A protein is much slower than that of the B- and C-isoforms.

To determine whether this amino acid motif enables HvKCO1 to bind to different barley 14-3-3 proteins, biotinylated synthetic phospho-peptides representing the putative 14-3-3 binding motif of HvKCO1 were attached to a streptavidin-coated biacore chip. The interaction between the peptide and recombinant 14-3-3 isoforms (0.5 μM) was followed in real-time using the surface plasmon resonance (SPR) method. Superimposed

sensorgrams (Fig. 4) show that all three 14-3-3 isoforms interact with the phospho-peptide in the presence of millimolar concentrations of divalent cations, whereas addition of EDTA completely prevents the binding of 14-3-3 proteins. Note that the dissociation of 14-3-3A is much slower than the dissociation of 14-3-3B and C, both in Mg^{2+} and Ca^{2+} buffer.

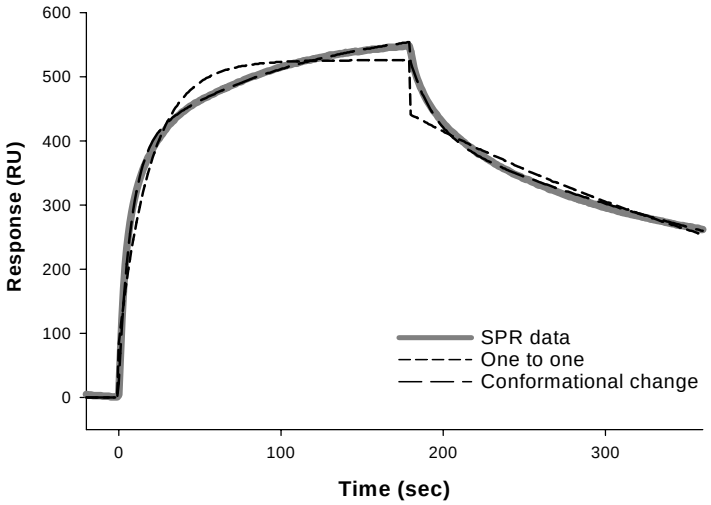

Figure 5. Example of surface plasmon resonance sensorgram fitted in BIAevaluation 3.1 with a Langmuir 1:1 model and the model for conformational change. Fitting with the Langmuir model does not result in a good fit, whereas the model that takes into account a conformational change fits the sensorgram very well.

To determine the affinity between the three 14-3-3 proteins and the HvKCO1 peptide, the kinetics of the sensorgrams were analyzed using BIAevaluation 3.1. First we used the Langmuir 1:1 model, which assumes a single binding site, but this model did not result in acceptable fits (Fig. 5). Athwal and Huber (2002) previously reported that 14-3-3 proteins are able to bind divalent cations in loop 8 and that this binding to the C-terminal tail is necessary for the inhibition of nitrate reductase (NR). Since our SPR data clearly show a requirement for divalent cations in the binding of 14-3-3 proteins to the HvKCO1 peptide, we analysed the sensorgrams using the conformational change model:

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\Leftrightarrow}} (AB) \underset{k_{d2}}{\overset{k_{a2}}{\Leftrightarrow}} (AB)^{*}, \qquad (1)$$

where k_{a1} and k_{d1} are the association and dissociation constant of peptide (A) and 14-3-3 (B) respectively and k_{a2} and k_{d2} represent the association and dissociation constants of a conformational change, for example caused by the movement of the C-terminal tail upon binding of divalent cations. The K_d can be calculated according to:

$$K_d = \frac{1}{(k_{a1}/k_{d1}) * (k_{a2}/k_{d2})} \quad (2)$$

These calculations show that both in Mg^{2+} and Ca^{2+} buffer 14-3-3A has the highest affinity for the HvKCO1 peptide (Table 1), followed by 14-3-3C and B. Thus, all barley 14-3-3 isoforms are able to bind to HvKCO1 with relatively high affinity in the presence of divalent cations, with slightly higher affinities in Ca^{2+} buffer as compared to Mg^{2+} buffer.

	Ca^{2+}	Mg^{2+}
14-3-3A	27.5	39.8
14-3-3B	48.3	91.7
14-3-3C	39.4	82.0

Table 1 Kinetic binding constants for the different 14-3-3 isoforms to the HvKCO1 peptide**.** Kinetic binding constants (nM) in Ca^{2+} and Mg^{2+} buffer, as determined by fitting the conformational change model on SPR data as described in Figure 5, show 14-3-3A has the highest affinity for HvKCO1, followed by that of the B- and C-isoforms.

Clear 14-3-3 isoform specificity in the regulation of the SV channel

In order to address the question whether the affinity of the three isoforms for the HvKCO1 peptide is reflected in their effectiveness of SV current inhibition, the activity and the properties of the SV channel was measured in a series of patch-clamp experiments. Recombinant 14-3-3 protein (100 nM) was added to the cytosolic side of barley mesophyll vacuoles (Fig. 6). Addition of 14-3-3B resulted in a decrease of the SV current by 30 % ± 1.9 as compared to the current under control conditions. A more drastic effect was observed upon addition of 14-3-3C, which inhibited the SV current by 64 % ± 3.1.

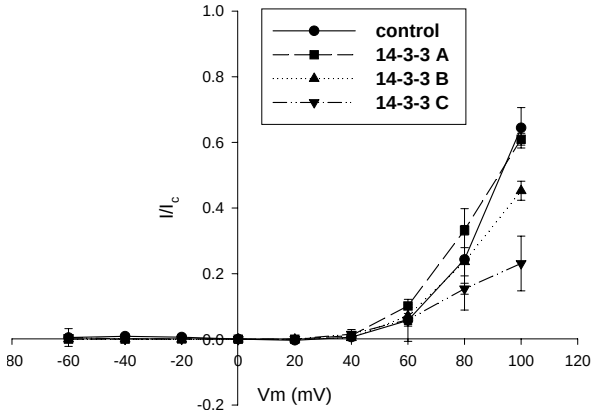

Figure 6. Current-voltage relationship of the time-dependent whole-vacuole SV channel current. Specific current (*I*) was measured at *t* = 20 min after application of buffer (control) or buffer plus 100 nM barley 14-3-3 isoforms A, B or C. The current at *t* = 20 min is shown in relation to the current (I_c) as measured just before addition of buffer or 14-3-3 protein to the bath: I/I_c. Currents are plotted against the activating voltages, ranging from +100 mV to -60 mV (holding potential is 0 mV) (*n* = 3, ± S.D.).

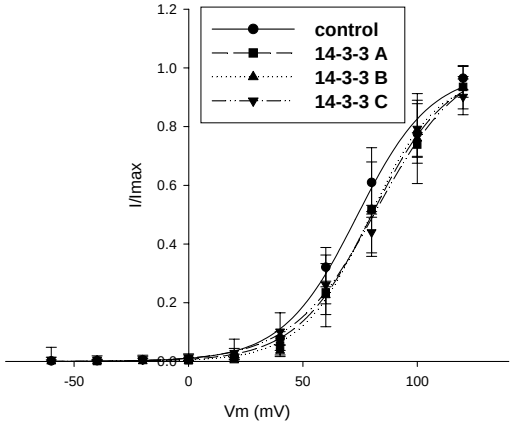

Figure 7. The different 14-3-3 isoforms do not have a significant effect on the voltage sensitivity of the SV channel, although they differ markedly in their effect on the current magnitude. Tail current analysis of the voltage sensitivity of the whole-vacuole SV current was performed by stepping the holding potential (0 mV) to activating potentials ranging from +100 to -60 mV (in steps of 20 mV). Tail current *I*, was determined after stepping the voltage from the activating potential to -60 mV. The voltage sensitivity was measured in the absence (control) or presence of 14-3-3 isoforms A, B and C, respectively 10 min upon the application. The data points were fitted by a Boltzmann distribution (*n* = 3; ± SD).

The effect of the 14-3-3 isoform with the highest affinity for the phsophorylated HvKCO1 peptide, 14-3-3A, on the SV current was only marginal; a reduction of 5 % (± 0.2). The voltage sensitivity of the channel, as determined from the tail currents like described before (Van den Wijngaard et al., 2001), was unaffected by the addition of 14-3-3 (Fig. 7).

Discussion

The KCO1 family contains a canonical 14-3-3 binding motif

Recent studies point to 14-3-3 regulation of vacuolar and plasma membrane K^+ channels as a recurrent theme in plant physiology (Saalbach et al., 1997; Booij et al., 1999; Van den Wijngaard et al., 2001; Bunney et al., 2002; De Boer, 2002; Van den Wijngaard et al., 2005). In support of this notion, specific 14-3-3 isoforms were shown to be differentially expressed upon K^+ starvation in *Arabidopsis* seedlings (Kang et al., 2004). The identification of AtKCO1 as a component of the SV channel (Schönknecht et al., 2002) prompted this study into the molecular basis of 14-3-3 regulation of this ubiquitously expressed channel, since it was shown that the SV channel in barley mesophyll cells is inhibited by 14-3-3B (Van den Wijngaard et al., 2001). Therefore, we isolated and sequenced the barley orthologue of *AtKCO1*, *HvKCO1*. The expression pattern and the phylogenetic analysis confirm *HvKCO1* is the barley equivalent of *AtKCO1*. The amino acid sequence of all known KCO1 orthologues contain a canonical 14-3-3 binding motif (RRxxS/TxP) in the N-terminal part of the protein, partly in the previously described KR-rich region (Czempinski et al., 1997). This binding motif is conserved in all known orthologues of KCO1 (Fig. 3) but not present in other members of the KCO family, what suggests that this may be a functional motif.

Functional expression of KCO1 in *Xenopus* oocytes has proven difficult so far. One reason for this might be that forward transport of the channel complex from the endoplasmic reticulum to the plasma membrane does not function properly in *Xenopus* oocytes. Ion channels with dibasic retention motifs are subject to retrograde transport to the ER by COPI-coated vesicles (O'Kelly et al., 2002). Recently, it was shown that

binding of 14-3-3 to these channels favours release from the ER and forward transport to the plasma membrane, because 14-3-3 masks the retention/retrieval motif, thus preventing the binding of the COPI-complex that mediates retention (Rajan et al., 2002). In some channel proteins the dibasic (RR or RK) retention motif (bold/italics) and the 14-3-3 binding motif (underlined, phosphor-serine marked) are very close: e.g. ***RR***SS^{P}V in KCNK3/9 K^+ channels (O'Kelly et al., 2002), ***RR***KS^{P}V in the two-pore domain K^+ channel TASK3 (Rajan et al., 2002) or ***RR***RSRS^{P}CR in the invariant chain protein lip35 (O'Kelly et al., 2002). The KCO1 proteins all contain a putative retention motif in the vicinity of the 14-3-3 interaction domain, ***KR***F***RR***SRS^{P}APR (HvKCO1) and it will be very interesting to study whether the expression of a mutated version of KCO1 lacking the putative retention signals results in measurable ion currents.

14-3-3 interacts with KCO1 in a divalent cation dependent manner

Surface plasmon resonance has proven to be a useful method in analysing 14-3-3 interactions, using both complete proteins (Masters et al., 1999; Van Hemert et al., 2003) and peptides representing the 14-3-3 binding domain (Muslin et al., 1996; Toroser et al., 1998; Athwal et al., 2000; Rosenquist et al., 2000) as a ligand. Since HvKCO1 is a membrane protein, and thus has hydrophobic regions, we chose to use the peptide derived from the putative 14-3-3 interaction domain (with phosphorylated serine-36) for the analysis of interaction with three barley 14-3-3 proteins. The results clearly show that the 14-3-3 proteins are able to bind with high affinity to this region of HvKCO1 in the presence of divalent cations. Interestingly, the absence of divalent cations completely prevents binding (Fig. 4).

The necessity for divalent cations in 14-3-3/target interaction is well known for nitrate reductase (Athwal et al., 1998) and sucrose-phosphate synthase (Toroser et al., 1998), where a conformational change of 14-3-3 upon binding of divalent cations (Lu et al., 1994; Athwal and Huber, 2002) allows interaction with the target. Amino acid substitutions in loop 8 of *Arabidopsis* 14-3-3ω altered the sensitivity for Mg^{2+} (Athwal and Huber, 2002), locating the divalent cation dependence to the putative EF-hand (Lu et al., 1994). However, not all 14-3-3 interactions are dependent on divalent cations. Binding

of 14-3-3 to 13-lipoxygenase is unaffected by Mg^{2+} (Holtman et al., 2000), while the interaction between 14-3-3 and phosphorylated non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase is even disrupted by Mg^{2+} (Bustos and Iglesias, 2003).

If 14-3-3 inhibits the SV current through the KCO1 component, the dependence of this interaction upon divalent cations may be of interest in the view of the fact that the activity of the SV channel is also strongly dependent upon divalent cations (Hedrich and Neher, 1987; Carpaneto et al., 2001; Van den Wijngaard et al., 2001). When functioning as a calcium induced calcium release channel (CICR) (Ward and Schroeder, 1994; Miedema et al., 2003), Ca^{2+} entering the cytosol from the vacuole will further activate the SV channel. However, a local increase in the Ca^{2+} concentration near the cytosolic side of the channel will favour the binding of 14-3-3 proteins, thereby reducing SV channel activity and Ca^{2+} release. In order to escape completely from this oscillation, channel phosphorylation followed by 14-3-3 binding may function as a full 'break'.

14-3-3 binding to HvKCO1-peptide does not correlate with SV inhibition by 14-3-3

The electrophysiological data presented here clearly show that 14-3-3B and C, but not 14-3-3A, are able to reduce the SV current (Fig. 6). None of the 14-3-3 isoforms changed the voltage sensitivity of the SV channel (Fig. 7), this unlike e.g. the effect of 14-3-3 proteins on the Drosophila Slowpoke calcium-dependent K^+ channel (Zhou et al., 1999) and the human ether-a-go-go (HERG) channel (Kagan et al., 2002). For plants a picture is emerging showing that 14-3-3 affects the pool of voltage activatable channels, rather than changing the gating properties (Booij et al., 1999; Van den Wijngaard et al., 2001; Van den Wijngaard et al., 2005). It is interesting that the SPR analysis indicates that all three isoforms bind to the HvKCO1, with 14-3-3A having the highest affinity (Table 1), whereas 14-3-3A is ineffective in reducing the SV current. In contrast, a good correlation was reported for the inhibition of nitrate reductase activity by five 14-3-3 isoforms and binding of the phospho-peptide derived from NR to these same 14-3-3 isoforms (Bachmann et al., 1996a). This was corroborated using an SPR analysis as described here, with barley NR phospho-peptide, barley NR activity and the three barley 14-3-3 isoforms (Sinnige et al., 2005a).

The reason for this discrepancy is not clear, but it might be that, i) a domain in the HvKCO1 protein other than the 14-3-3 interaction motif specifies the affinity for the full protein, ii) HvKCO1 may be only one component of the SV channel, as suggested by Schönknecht (2002), whereas a second subunit may have different affinity for the 14-3-3 isoforms. One indication that KCO1 may be only a component of the SV current, is that all KCO channels contain a GYGD motif, what usually results in K^+ selective rather than non-selective channels, as the SV channel (Ivashikina and Hedrich, 2005). In these cases, inhibition of the SV current could require interaction with both monomeric subunits within a single 14-3-3 dimer and function according to the 'gatekeeper' model as suggested for the 14-3-3 regulation of Raf-1 (Yaffe, 2002). In addition, the 14-3-3 molecules are capable of forming both homo- and heterodimers (Jones et al., 1995; Wu et al., 1997; Paul et al., 2005), what could imply that a 14-3-3 heterodimer (with 14-3-3A binding to the currently investigated binding motif and e.g. 14-3-3C to a putative second binding site) is responsible for inhibition of the SV current *in vivo*. Expression of KCO1 proteins in *kco1* knock-out plants, with mutations in the 14-3-3 interaction site and the putative di-basic retention signal will provide further insight in the role of these domains for the functioning of KCO1 in the physiology of the plant.

Experimental procedures

Isolation and characterisation of HvKCO1

AtKCO1 was used to search the BLAST results of a barley EST library (http://pgrc.ipk-gatersleben.de/cr-est/index.php) and clone HK03F06u was identified as a KCO family member. Using primary leaf of *Hordeum vulgare* cv. Alexis (Josef Breun Saatzucht, Herzogenaurach, Germany), the cDNA fragment was extended to the 5' using the Universal GenomeWalker Kit (BD Biosciences, Palo Alto, CA, USA) with two gene specific primers (1: 5'-CTCCACGAGATAATCCGCCGACTTG-3' and 2: 5'-CGACGACGCCCGCTAGCAGG-3'). The complete cDNA sequence of *HvKCO1* was obtained using 3' RACE (BD Biosciences) with gene specific primers (3: 5'-GCGGGCGTCGTCGTCTTTTACC-3' and 4: 5'-GCTGCTCGCTTGTGTGTTCGTC-

3'). For expression experiments, total RNA from barley tissues was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was produced using SuperScript reverse transcriptase (Invitrogen). Barley radicles were isolated and maintained as described previously (Van den Wijngaard et al., 2005). Quantitative RT-PCR (DNA Engine Opticon, MJ Research, Inc., Waltham, MA, USA) was done using primers KCOf (5'-GTTTGAGAAACTCGATGTTGACCA-3'), KCOr (5'-CAAGCCCATATGTTCATC ACTGAC-3'), Actinf (5'-GTATGGAAACATCGTGCTCAGTGG-3') and Actinr (5'-CTTGATCTTCATGCTGCTCGGA-3'). GenBank accession nos.: AtKCO1 (X97323), HvKCO1 (AY770627), Actin (AY145451). All kits were used according to the manufacturers protocol.

Expression and purification of recombinant 14-3-3 proteins

Barley 14-3-3 isoforms A, B and C (GenBank accession nos. X62388, X93170 and Y14200, respectively) were cloned into the pRSETC vector (Invitrogen) and the orientation and reading frame checked by sequencing. Transformed BL21 (DE3) pLysS cells (Invitrogen) were grown O/N at 37°C in 2 x YT, 1 % glucose and 50 µg/ml ampicillin until the OD_{600} was 0.8 - 0.9. Expression was induced by replacing the medium by 2 x YT, 1 mM isopropyl-β-D-thiogalactoside (AppliChem, Darmstadt, Germany) and 50 µg/ml ampicillin and incubation at 28°C for 4 h. Cells were pelleted at 5000 *g* for 15 min and dissolved in 30 ml 20 mM Hepes/KOH pH 7.5, 0.5 M NaCl, 1 mM PMSF and l0 mM imidazol (Merck, Darmstadt, Germany) at 4°C. Once dissolved, 1 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and 4 µg/ml deoxyribonuclease 1 (Sigma-Aldrich) were added and incubated for 30 min. The cell lysate was centrifuged for 30 min at 100 000 *g* and supernatant filtered through a 0.45 µM filter (Schleicher & Schuell, Dassel, Germany). The filtrate was applied to a Ni^{2+} charged HiTrap Chelating HP column (Amersham Pharmacia, Uppsala, Sweden) and subjected to a 10 mM to 500 mM imidazol gradient. The 14-3-3 peak was pooled, desalted to 1 mM Hepes/KOH pH 7.5 using a HiPrep 26/10 desalting column (Amersham) and concentrated by freeze drying. Quantity and quality of the recombinant proteins were checked using the Bradford protein assay (BioRad, Hercules, CA, USA), Coomassie stained SDS-PAGE gel and far UV circular

dichroism (not shown).

Surface plasmon resonance

Surface plasmon resonance experiments were performed with a Biacore 2000 (Biacore, Uppsala, Sweden). A streptavidin coated sensor chip (Biacore) was activated with 1 M NaCl, 50 mM NaOH, flowpath 2 was coated at 5 µl/min with 1000 response units of KCO-P peptide (Biotin-GAKRFRRSRS^{p}APRSE) (Ansynth, Roosendaal, The Netherlands) in immobilization buffer (10 mM KAc, pH 6 with HAc). Measurements were done in running buffer (20 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 25 mM NaCl and 15 mM imidazol), at 25°C using a flowrate of 10 µl/min. An association step of 3 min, with a 14-3-3 isoform diluted in running buffer, was followed by a dissociation step of 3 min in running buffer. The chip was regenerated between runs using 0.5 % SDS (w/v), 50 mM NaCl pH 6.8. The data was processed using BIAevaluation 3.1. (Biacore).

Vacuole isolation

Barley leaf mesophyll vacuoles were isolated as described (Van den Wijngaard et al., 2001). Briefly, the epidermal layer was stripped from primary barley leaves. The mesophyll cell layer was exposed to an enzyme solution containing 1 mM $CaCl_2$, 500 mM sorbitol, 0.05% (w/v) polyvinylpyrrolidone, 15 mM MES/Tris pH 5.5, 0.2% (w/v) bovine serum albumin, 1% (w/v) cellulase, 0.5% (w/v) Macerozym, 0.01% (w/v) pectolyase and agitated for 30 min at room temperature. Protoplasts were released by gently pressing the leaves on a mesh and suspended in 100 mM KCl, 10 mM HEPES/KOH pH 7.5, 10 mM $MgCl_2$, sorbitol to 525 mOsm/kg. For the patch-clamp experiments vacuoles from spontaneously bursting mesophyll protoplasts were used.

Patch-clamp recordings

Patch-clamp recordings were performed in the whole-vacuole mode with an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA) and low-pass filtered using a four pole Bessel filter (internal filter of the Axopatch). The data were digitized using a Digidata 1320A (Axon Instruments, USA) and analyzed with pClamp 8.0 (Axon Instruments,USA). Patch pipettes were pulled from Kimax-51 glass (Kimble Products, USA) and briefly fire-polished. Pipettes were filled with 100 mM KCl, 10 mM HEPES/KOH pH 7.5, 5 mM $MgCl_2$, sorbitol to 525 mOsm/kg. The bath was flushed with bath solution (100 mM KCl, 10 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 200 µM $CaCl_2$, sorbitol to 525 mosm/kg) using a peristaltic pump at 0.2 ml/min. Perfusion was stopped before 14-3-3 protein was added to the bath and a number of pulse protocols were run to test whether the SV current remained stable. Then, 5 µl 14-3-3 was added to the bath (volume 0.2 ml) from a stock solution (0.25 mg/ml in 10 mM HEPES/KOH, pH 7.5) giving a final concentration of 100 nM 14-3-3 protein. Before and after voltage test steps, vacuoles were held at a membrane potential of 0 mV; test voltages ranged from +100 mV to -60 mV (in steps of 20 mV). Tail currents were analysed with a protocol where the membrane voltage was stepped from activating voltages (ranging from +100 to -60 mV) to a voltage of -60 mV. Only measurements were taken where capacitance and serial resistance changes during the measurement were below 10%.

Acknowledgements

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Chapter 3

Single amino acid variation in barley 14-3-3 proteins leads to functional isoform specificity in the regulation of nitrate reductase

Published in collaboration with Ilja Roobeek, Tom D. Bunney, Antonie J.W.G. Visser, Jos N.M. Mol and Albertus H. de Boer

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Summary

The highly conserved family of 14-3-3 proteins function in the regulation of a wide variety of cellular processes. The presence of multiple 14-3-3 isoforms and the diversity of cellular processes regulated by 14-3-3 proteins suggest functional isoform specificity of 14-3-3 isoforms in the regulation of target proteins. Indeed, several studies observed differences in affinity and functionality of 14-3-3 isoforms. However, the structural variation by which isoform specificity is accomplished remains unclear. Because other reports suggest that specificity is found in differential expression and availability of 14-3-3 isoforms, we used the nitrate reductase (NR) model system to analyse the availability and functionality of the three barley 14-3-3 isoforms. We found that 14-3-3C is unavailable in dark harvested barley leaf extract and 14-3-3A is functionally not capable to efficiently inhibit NR activity, leaving 14-3-3B as the only characterized isoform able to regulate NR in barley. Further, using site directed mutagenesis, we identified a single amino acid variation (Gly versus Ser) in loop 8 of the 14-3-3 proteins that plays an important role in the observed isoform specificity. Mutating the Gly residue of 14-3-3A to the alternative residue, as found in 14-3-3B and 14-3-3C, turned it into a potent inhibitor of NR activity. Using surface plasmon resonance we show that the ability of 14-3-3A and the mutated version to inhibit NR activity correlates well with their binding affinity for the 14-3-3 binding motif in the NR protein, indicating involvement of this residue in ligand discrimination. These results suggest that both availability of 14-3-3 isoforms as well as binding affinity determine isoform specific regulation of NR activity.

Introduction

Members of the highly conserved family of 14-3-3 proteins play a key role in cell signalling. To date, over 100 binding partners of 14-3-3 proteins have been identified that are involved in a wide array of functions: these include controlling metabolic enzymes (Huber et al., 2002), cell cycle control (Van Hemert et al., 2001), ion transport (Bunney et al., 2002; De Boer, 2002), gene expression (Eckardt, 2001), kinase activity (Camoni et al., 1998) and the assembly and localization of protein complexes (Rajan et al., 2002). Multiple isoforms of 14-3-3 proteins are found in all tested eukaryotic organisms, ranging from two isoforms in yeast and Drosophila to twelve expressed isoforms in *Arabidopsis* (Rosenquist et al., 2001). The 14-3-3 proteins act as dimers and their ability to form both homo- and heterodimers creates a large population of dimeric molecules.

The diversity in 14-3-3 molecules and the question whether they are functionally redundant or not is one of the major issues in 14-3-3 biology. The presence of so many different 14-3-3 isoforms in plants suggests functional isoform specificity in the regulation of target proteins. However, residues in the amphipathic groove that are involved in ligand binding are highly conserved among 14-3-3 isoforms (Zhang et al., 1997; Petosa et al., 1998; Wang et al., 1998). Alternatively, some reports suggest that 14-3-3 isoforms are redundant in their ability to act on target proteins and specificity is found in expression patterns and availability of 14-3-3 proteins (Roberts, 2000; Roberts and de Bruxelles, 2002; Zuk et al., 2005). Indeed, in peptide screens, different 14-3-3 isoforms seem to bind with similar affinities (Muslin et al., 1996; Yaffe et al., 1997) and several *Arabidopsis* 14-3-3 isoforms can complement the lethal yeast BMH1 BMH2 double disruption what indicates redundancy (Van Heusden et al., 1996; Kuromori and Yamamoto, 2000). Further supporting this theory, several reports show differential expression (Testerink et al., 1999; Roberts and de Bruxelles, 2002; Sehnke et al., 2002b; Maraschin et al., 2003b; Qi et al., 2005) and subcellular localization of 14-3-3 isoforms (Martin et al., 1994; Bunney et al., 2001; Van Hemert et al., 2004; Paul et al., 2005), affecting the availability of 14-3-3 isoforms.

On the other hand, (over-)expression of 14-3-3 isoforms in a heterologous system like yeast may override subtle differences in affinity and the subcellular localization of 14-3-3 proteins was recently shown to be mainly target driven and is therefore rather an indication for isoform specific interactions (Paul et al., 2005). Further, in recent years clear evidence emerged for 14-3-3 isoform-specific protein-protein interactions. By analysing several 14-3-3 isoforms in the same experimental system, clear differences in affinity and functionality were observed in the regulation of nitrate reductase (NR) (Bachmann et al., 1996a), the plasma membrane H^+-ATPase (Rosenquist et al., 2000; Emi et al., 2001; Alsterfjord et al., 2004), the SV channel (Sinnige et al., 2005b) and sucrose-phosphate synthase (Bornke, 2005). For example, Bachmann et al. (1996a) showed 14-3-3ω is a much more potent inhibitor of NR activity compared to four other tested 14-3-3 isoforms. These differences correlate with the ability of the 14-3-3 isoforms to bind a synthetic NR peptide, providing evidence for isoform specific protein-protein interaction. However, the molecular basis for this observed isoform specificity remains unclear.

We have used the NR model system to study 14-3-3 isoform specificity in barley. The reduction of nitrate to nitrite by NR is an important step in nitrogen assimilation. Nitrite is further reduced to ammonia by nitrite reductase, of which the activity depends on electrons donated by photosynthesis. To prevent accumulation of the toxic nitrite, NR is rapidly phosphorylated upon darkness and consequent 14-3-3 binding inhibits NR activity (Huber et al., 1996; Kaiser and Huber, 2001; Comparot et al., 2003). At pH 7.5, inhibition of NR by 14-3-3 proteins requires the presence of certain polyamines or millimolar concentrations of divalent cations (Bachmann et al., 1996b; Athwal et al., 1998; Athwal and Huber, 2002). Divalent cations seem to act through interaction with an EF hand-like structure in loop 8 of the 14-3-3 proteins, where upon binding of divalent cations a conformational change in the C-terminal tail is induced (Lu et al., 1994). Mutated 14-3-3 proteins, in which conserved amino acids in this EF hand-like structure were altered, indeed affected the divalent cation dependence of NR inhibition (Athwal and Huber, 2002). Further, partial removal of the C-terminal tail, which might function as an auto-inhibitor within the ligand binding groove of the 14-3-3 protein (Kubala et al., 2004), increased the affinity for target proteins (Truong et al., 2002; Shen et al., 2003).

Here, we report that, both due to differential expression and functional isoform specificity, barley 14-3-3B is the main candidate to regulate foliage NR in barley. Further, using directed mutagenesis, we show that a natural varying amino acid in loop 8 of 14-3-3 proteins plays an important role in isoform specific target recognition in the regulation of NR activity.

Results

The discussion on 14-3-3 isoform specificity focuses on functionality versus availability of the 14-3-3 proteins. In plants, functional differences between 14-3-3 isoforms were first shown in the regulation of NR (Bachmann et al., 1996a). Therefore, we adapted an NR extraction method previously described by Bachmann et al. (1995) to determine both the presence of individual 14-3-3 isoforms and the ability of the 14-3-3 isoforms to inhibit NR activity. Dark harvested barley leaf extract was subjected to anion-exchange chromatography to separate NR activity from inhibiting 14-3-3 proteins (Fig. 1). The NR activity of the eluted fractions was determined using an NR assay and fractions with peak activity, fractions 24 to 28, were pooled to form the NR extract. To identify the fractions in which the 14-3-3 proteins eluted, all fractions were mixed with an aliquot of the NR extract and again assayed for NR activity. An expected increase in NR activity was seen in fractions where NR eluted. This was followed by an inhibition in NR activity, in fractions 32 to 40, indicating that the inhibiting 14-3-3 proteins elute in these fractions.

Thus far, three 14-3-3 isoforms have been described in barley and the presence of these isoforms in the eluted fractions was tested using isoform-specific antibodies (Fig. 2). Indeed, 14-3-3 proteins were found in the fractions where inhibition of NR activity was located. Interestingly, 14-3-3A and 14-3-3B were relatively abundant where almost no 14-3-3C was present in the eluted fractions. Further, the peak of 14-3-3B was detected in later-eluting fractions compared to 14-3-3A. Inhibition of NR activity was highest in fractions 34 to 37 (Fig. 1), what corresponds with the fractions in which 14-3-3B was located. These results suggest that NR activity in barley is inhibited by 14-3-3B.

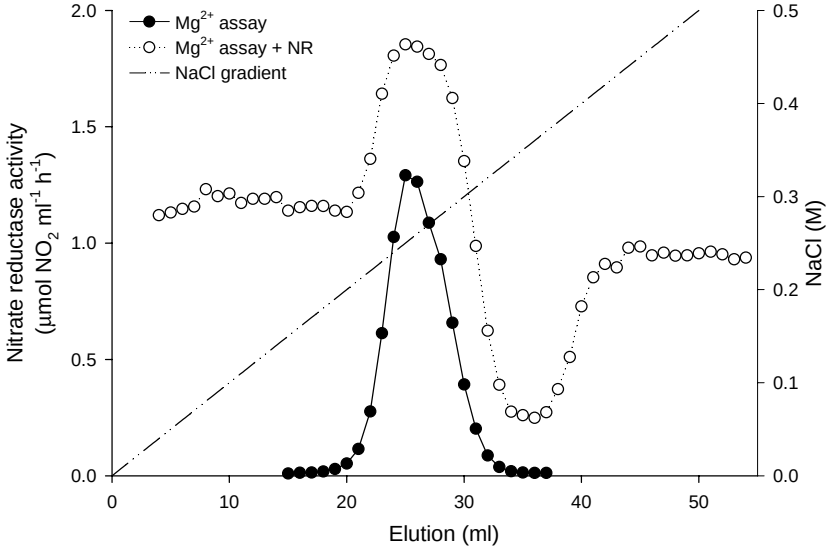

Figure 1. Identification of NR and 14-3-3 containing fractions. Dark harvested barley leaf protein extract was subjected to anion exchange chromatography and eluted fractions were assayed for NR activity in the presence of 5 mM Mg^{2+} (○). Fractions of peak activity were pooled and an aliquot of this active fraction was mixed with the eluted fractions. These mixtures were again assayed for NR activity in the presence of Mg^{2+} to locate the inhibiting 14-3-3 proteins (●).

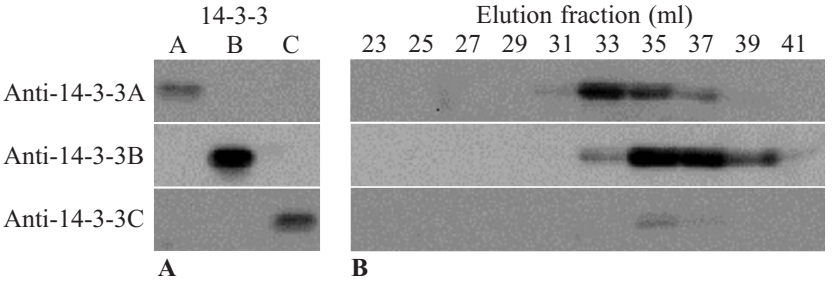

Figure 2. Barley leaf extract contains 14-3-3A and 14-3-3B. (A) Isoform specific antibodies. Purified histidine labeled 14-3-3 isoforms (0.5 pmol) were subjected to SDS-PAGE and blotted on nitrocellulose membrane. Blots were hybridized with anti-14-3-3A, B or C to show the specificity of the 14-3-3 antibodies (Testerink et al., 1999). (B) Presence of 14-3-3 isoforms in barley leaf extract. Anion exchange chromatography of dark harvested barley leaf extract resulted in fractions containing NR activity and NR inhibiting fractions as described in Figure 1. These fractions were subjected to SDS-PAGE, blotted on nitrocellulose membrane and hybridized with anti-14-3-3A, B or C to show the presence of 14-3-3 isoforms in barley leaf extract. Fractions pooled to form the NR extract (24 to 28) are free of 14-3-3 isoforms.

The ability of individual 14-3-3 isoforms to inhibit NR activity was tested using recombinant 14-3-3 proteins (Fig. 3). In line with the observation that the distribution of 14-3-3A does not correlate with the peak inhibition of NR activity and previously reported differences in NR inhibition by *Arabidopsis* 14-3-3 isoforms (Bachmann et al., 1996a), 14-3-3A is a very poor inhibitor of NR activity as compared to 14-3-3B and 14-3-3C (Fig. 3). However, the isoform that was hardly present in the extract, 14-3-3C, was most effective in inhibiting NR activity, closely followed by 14-3-3B. Further, all 14-3-3 isoforms show a reduced inhibitory effect in the absence of divalent cations (Fig. 3c) as previously reported for *Arabidopsis* 14-3-3ω (Bachmann et al., 1996b; Athwal et al., 1998; Athwal and Huber, 2002). Divalent cations are thought to interact with an EF hand-like structure in loop 8 of the 14-3-3 proteins, causing a conformational change through which the 14-3-3 binding groove becomes more accessible for target proteins. Here, 14-3-3A completely loses all inhibitory effect, where 14-3-3B and to a lesser extent 14-3-3C are reduced in their potency to inhibit NR activity.

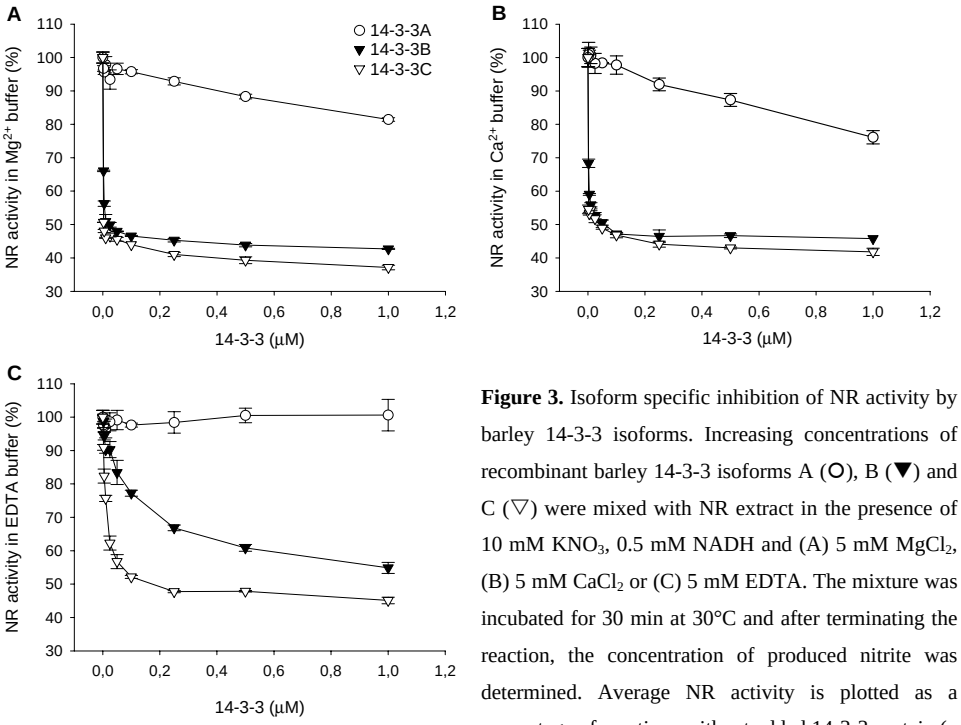

Figure 3. Isoform specific inhibition of NR activity by barley 14-3-3 isoforms. Increasing concentrations of recombinant barley 14-3-3 isoforms A (○), B (▼) and C (▽) were mixed with NR extract in the presence of 10 mM KNO_3, 0.5 mM NADH and (A) 5 mM $MgCl_2$, (B) 5 mM $CaCl_2$ or (C) 5 mM EDTA. The mixture was incubated for 30 min at 30°C and after terminating the reaction, the concentration of produced nitrite was determined. Average NR activity is plotted as a percentage of reactions without added 14-3-3 protein (*n* = 3, ±SD).

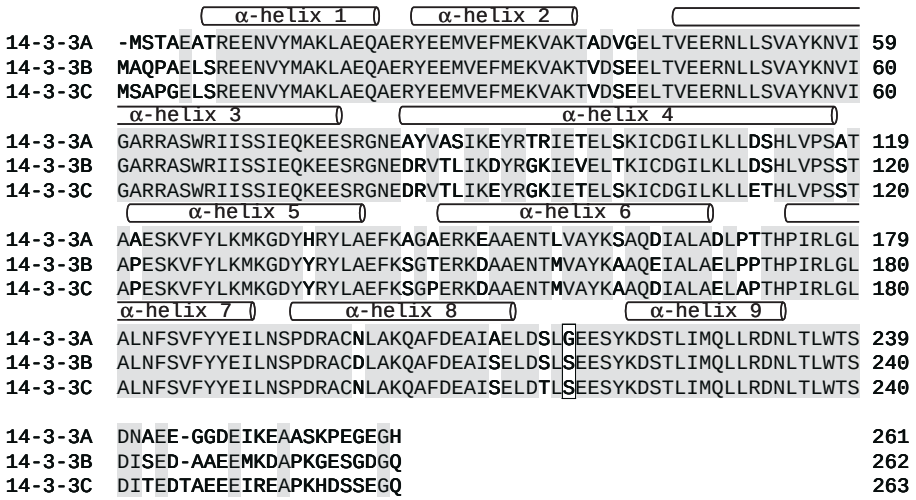

Figure 4. Sequence conservation among barley 14-3-3 isoforms. Amino acid sequence alignment of the three known barley 14-3-3 isoforms. Identical amino acids are marked in gray and predicted α-helices (1-9) are indicated above the sequence. 14-3-3B and 14-3-3C have a similarity of 91% and both have 82% similarity with 14-3-3A. The critical residue in loop 8 that is converted into a serine in 14-3-3A^{G216S} is depicted in bold and boxed.

Although 14-3-3 proteins show a high degree of similarity at the protein level, 14-3-3B and 14-3-3C share 91% similarity and both have 82% similarity with 14-3-3A (Fig. 4), the results in Fig. 3 show clearly that 14-3-3A differs functionally from 14-3-3B and 14-3-3C. Previous reports have suggested a role for the relatively more divergent C-terminal tail in ligand discrimination (Athwal and Huber, 2002; Shen et al., 2003) possibly functioning as an auto-inhibitor of 14-3-3 action (Truong et al., 2002; Shen et al., 2003) by occupying the ligand binding groove (Kubala et al., 2004; Silhan et al., 2004). The amino acids in loop 8 form an EF hand-like structure (Lu et al., 1994), which is reported to affect the conformation of helix 9 and the C-terminal tail in 14-3-3 proteins (Lu et al., 1994; Athwal and Huber, 2002; Kubala et al., 2004). Therefore, it is likely that structural difference in this region affects the functionality of the protein. Indeed, directed mutagenesis of conserved amino acids in loop 8 affected the ability of 14-3-3ω to inhibit NR (Athwal and Huber, 2002) and three unique natural varying amino acids in loop 8 of mammalian 14-3-3σ were recently shown to cause functional specificity (Wilker et al., 2005). In the loop 8

region of 14-3-3A only two amino acid residues, Ala-210 and Gly-216, vary from both 14-3-3B and 14-3-3C (Fig 4) of which Gly-216 is the central residue in the proposed EF hand-like structure. Using site-directed mutagenesis we produced 14-3-3A^{G216S} to investigate whether this amino acid substitution would affect the inhibiting effect of 14-3-3A on NR activity. Although the mutation is located in a loop and therefore unlikely to affect the secondary structure, circular dichroism (CD) spectroscopy was used to make sure the secondary structure of 14-3-3A^{G216S} was unaffected by the mutation (Fig. 5). The obtained spectra are comparable to previously reported spectra of 14-3-3 proteins (Wang et al., 1998; Athwal and Huber, 2002) and show that α-helices are the major component in the secondary structure. The α-helical content of 14-3-3A is slightly lower than observed for 14-3-3B and 14-3-3C, possibly reflecting some structural differences between the different isoforms. More importantly, the CD spectra of 14-3-3A and 14-3-3A^{G216S} were essentially identical, showing the mutation did not affect the secondary structure.

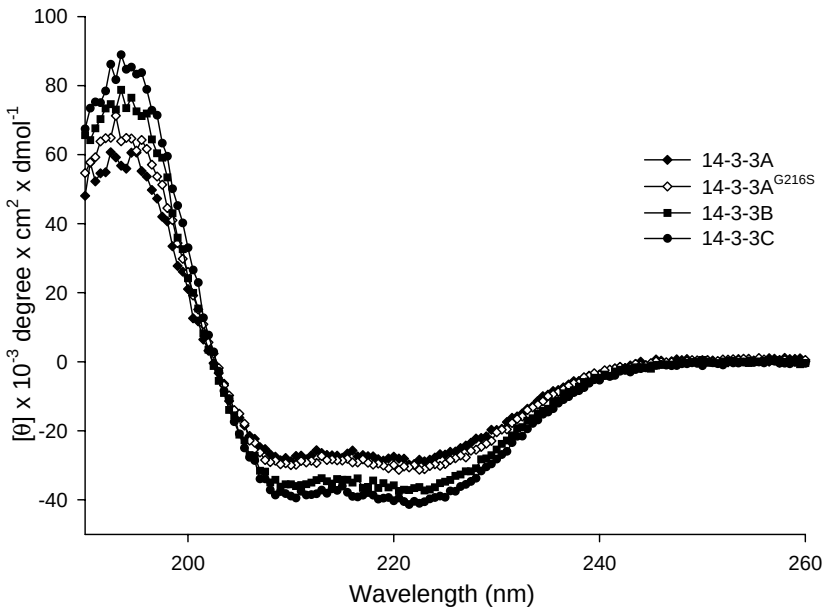

Figure 5. Far-UV CD spectra of WT and mutant recombinant 14-3-3 proteins. Far-UV CD spectra of recombinant 14-3-3 proteins were generated on a Jasco J-715 spectropolarimeter at 20ºC using 0.1 mg/ml protein in 5 mM Na-phosphate pH 7.5. The molar ellipticity, [θ], is the average of 5 independent scans and buffer blank spectra were substracted to compensate for solvent contribution.

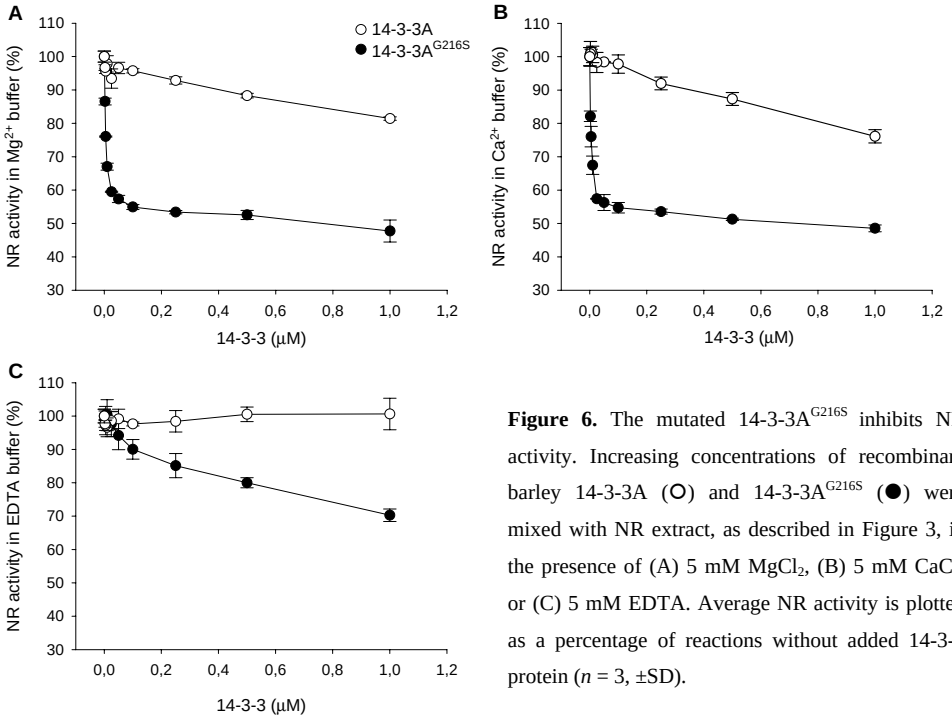

Figure 6. The mutated 14-3-3A^{G216S} inhibits NR activity. Increasing concentrations of recombinant barley 14-3-3A (○) and 14-3-3A^{G216S} (●) were mixed with NR extract, as described in Figure 3, in the presence of (A) 5 mM $MgCl_2$, (B) 5 mM $CaCl_2$ or (C) 5 mM EDTA. Average NR activity is plotted as a percentage of reactions without added 14-3-3 protein (n = 3, ±SD).

Both in the presence and absence of divalent cations the inhibitory effect of 14-3-3A^{G216S} on NR activity is dramatically increased as compared to 14-3-3A (Fig. 6). The concentration of 14-3-3 required to reach half maximal inhibition, K_i, in the presence of Mg^{2+} is 5.4 nM for 14-3-3A^{G216S}, which is approximately a 1000-fold lower as compared to 14-3-3A, and is comparable with 14-3-3B and 14-3-3C (Table 1). These results clearly show that Ser-216 increases the functionality of 14-3-3 proteins in NR inhibition and is a basis for isoform specific regulation by 14-3-3 proteins.

	Mg^{2+}	Ca^{2+}	EDTA
14-3-3A^{G216S}	5.4 nM	4.9 nM	404.6 nM
14-3-3A	4597.8 nM	2633.7 nM	N.D.
14-3-3B	1.5 nM	1.7 nM	121.6 nM
14-3-3C	0.5 nM	0.7 nM	11.7 nM

Table 1. Kinetic evaluation of the ability of the 14-3-3 proteins to inhibit NR. The enzymatic assays from Fig. 3 and Fig. 6 showing the inhibition of NR by the different 14-3-3 isoforms and the mutated 14-3-3A^{G216S} in the presence and absence of 5 mM Mg^{2+}/Ca^{2+}, were used to determine the concentration of 14-3-3 required to reach half maximal inhibition, K_i. N.D., not determined.

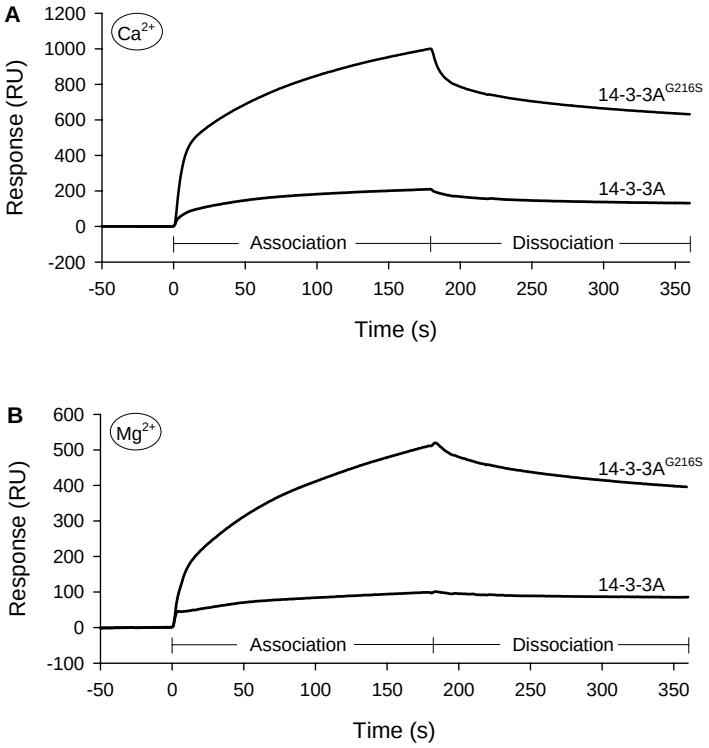

Figure 7. Mutation affects binding to target protein. Superimposed surface plasmon resonance sensorgrams generated on a Biacore 2000 in the presence of (A) 5 mM $CaCl_2$, (B) 5 mM $MgCl_2$. Sensorgrams show real time binding of recombinant 14-3-3A and the mutated 14-3-3A^{G216S} to immobilized phosphorylated NR representing synthetic peptide. Each curve shows three phases: the first phase represents equilibration with running buffer, the second phase shows binding of the 14-3-3 protein to the chip (association) and the third phase shows dissociation of 14-3-3 protein when only running buffer is passed over the chip (dissociation).

Bachmann et al. (1996a) previously showed that the ability of different 14-3-3 isoforms to inhibit NR correlates to their ability to bind to the target sequence. To determine whether the increased functionality of 14-3-3A^{G216S} reflects improved target recognition, biotinylated synthetic phospho-peptides representing the 14-3-3 binding motif of barley NR were attached to a streptavidin-coated Biacore chip. Interaction between the peptide and recombinant 14-3-3A/14-3-3A^{G216S} (0.5 μM) was followed in real-time using surface plasmon resonance. Superimposed sensorgrams (Fig. 7) show that, both in presence of 5 mM Ca^{2+} or 5 mM Mg^{2+}, the ability to bind NR is significantly enhanced in 14-3-3A^{G216S}.

This indicates Ser-216 is involved in recognizing NR. In the presence of 5 mM EDTA, no binding by 14-3-3A nor by 14-3-3A^{G216S} was observed (data not shown).

Discussion

The functional diversity of the family of 14-3-3 proteins in regulating cellular processes in plants has raised questions concerning the role of the different 14-3-3 isoforms. In contrast to the proposed functional redundancy of 14-3-3 isoforms (Roberts, 2000; Roberts and de Bruxelles, 2002; Zuk et al., 2005), clear functional differences between *Arabidopsis* 14-3-3 isoforms were observed in the regulation of NR (Bachmann et al., 1996a). Following that, functional isoform specificity of 14-3-3 proteins was observed in the regulation other 14-3-3 target proteins like the plasma membrane H^+-ATPase (Rosenquist et al., 2000; Emi et al., 2001; Alsterfjord et al., 2004), the SV channel (Sinnige et al., 2005b) and sucrose-phosphate synthase (Bornke, 2005). The subcellular localization of 14-3-3 isoforms, an argument used to support the functional redundancy theory, was recently shown to be mainly target driven and is therefore rather an indication for isoform specific interactions (Paul et al., 2005).

A confusing factor in the discussion on isoform specific functions versus differential availability is the shortage of reports in which multiple 14-3-3 isoforms are tested for both. In the present study we used one of the best-characterized roles of plant 14-3-3 proteins, the inhibition of NR, to investigate this. Here we demonstrate that NR activity in barley can be efficiently inhibited by recombinant 14-3-3B and 14-3-3C, but not by 14-3-3A (Fig. 3). This implies functional isoform specificity concerning 14-3-3A but also a potential functional redundancy of 14-3-3B and 14-3-3C in inhibiting NR. Immunolocalization studies in germinating barley embryos showed a differential expression of the three barley 14-3-3 isoforms (Testerink et al., 1999). 14-3-3A and 14-3-3B dominated expression in leaf tissue of germinating embryos while almost no 14-3-3C was detected. This resembles the presence of 14-3-3 isoforms in the extract from 1-week-old barley leaves (Fig. 2). Indeed, the difference between 14-3-3B and 14-3-3C in the regulation of NR is the availability of these isoforms, leaving 14-3-3B as the only characterized 14-3-3 isoform able to regulate NR in barley. Further, this provides an

example in which functional isoform specificity as well as differential availability determines the biological role for a given 14-3-3.

The question remains as to the cause of the functional difference that makes 14-3-3A such a poor inhibitor of NR activity as residues in the amphipathic groove that are involved in ligand binding are highly conserved among 14-3-3 proteins (Zhang et al., 1997; Petosa et al., 1998; Wang et al., 1998). Here we identified a natural varying single residue in loop 8, responsible for at least part of the isoform specificity of barley 14-3-3A. Mutating Gly-216 to Ser-216, as in 14-3-3B and 14-3-3C, restored the ability of 14-3-3A to inhibit NR (Fig. 6). Further, surface plasmon resonance shows that the mutated 14-3-3A^{G216S} increases the ability to interact with an NR representing peptide as compared to 14-3-3A, indicating that isoform specificity is found in ligand recognition (Fig. 7). Previously, directed mutagenesis of two conserved amino acids in this region was shown to affect the ability of 14-3-3ω to inhibit NR (Athwal and Huber, 2002) and three unique residues in loop 8 of the mammalian 14-3-3σ were recently shown to cause functional isoform specificity (Wilker et al., 2005). This loop 8 has been proposed to function in binding divalent cations through a putative EF-hand (Lu et al., 1994). Binding of divalent cations induces a conformational change and possibly alters the position of helix 9 and the C-terminal tail. Helix 9 is involved in the formation of the amphipathic groove in which 14-3-3 proteins bind their ligands (Petosa et al., 1998) and the C-terminal tail of 14-3-3 proteins may function in ligand discrimination (Athwal and Huber, 2002; Shen et al., 2003) by occupying the ligand binding groove (Kubala et al., 2004; Silhan et al., 2004) and function as an auto-inhibitor of 14-3-3 action (Truong et al., 2002; Shen et al., 2003). Possibly, mutating Gly-216 to Ser-216 in loop 8 of 14-3-3A induces a similar alteration in the position of helix 9 and the C-terminal tail, thereby increasing the accessibility of the binding groove. Alternatively, this mutated residue might function in ligand recognition as suggested for the three unique residues in loop 8 of 14-3-3σ (Wilker et al., 2005). In support of this, loop 8 of 14-3-3ξ was shown to make direct contact with part of its co-crystallized ligand (Obsil et al., 2001).

These results show that this natural varying amino acid serves a significant role in ligand discrimination and functional isoform specificity. Many other plant species contain one or more 14-3-3 isoform(s) with a Ser residue in the same position as barley 14-3-3B and 14-3-3C. In *Arabidopsis*, for example, this residue is present in 14-3-3ι. However, additional mutagenesis of natural variations between 14-3-3 isoforms is necessary to fully understand the observed functional differences between 14-3-3 isoforms. In analysing isoform specificity in NR inhibition, Bachmann et al. (1996a) tested five *Arabidopsis* 14-3-3 isoforms (ω, ψ, ϕ, χ, ν), all containing a Gly residue like 14-3-3A, and observed clear differences in their ability to inhibit NR. Moreover, although the K_i of 14-3-3A^{G216S} in inhibiting NR is approximately a 1000-fold lower as compared to 14-3-3A (Table 1), the affinity is still 10-fold less than observed for 14-3-3C. Therefore, other natural variations between 14-3-3 isoforms, like the C-terminal tail, must function in isoform specificity as well. Interestingly, we estimate, from the data provided by Bachmann et al. (1996a), that the K_i of the most potent tested inhibitor from *Arabidopsis*, 14-3-3ω, is approximately 0.6 μM. Barley 14-3-3C is, with a K_i of 0.5 nM (Table 1), approximately a 1000-fold more effective. This rather large difference begs the question whether a yet un-investigated *Arabidopsis* 14-3-3 isoform might have a higher affinity for NR. An obvious candidate would be 14-3-3ι since this isoform contains the Ser residue as found in barley 14-3-3 B and C. However, 14-3-3ι expression has thus far been mainly observed in flowers (Rosenquist et al., 2001) while NR is located in leaf tissue. This makes it unlikely that 14-3-3ι functions in inhibiting NR, as both capability and availability of 14-3-3 isoforms are shown to be crucial in regulating target proteins.

Experimental procedures

Purification of recombinant 14-3-3 proteins and site directed mutagenesis

Barley 14-3-3 isoforms A, B and C (GenBank accession nos. X62388, X93170 and Y14200, respectively) were cloned into the pRSETC vector (Invitrogen, Carlsbad, CA, USA) and the orientation and reading frame checked by sequencing. The mutated 14-3-3A^{G216S} was generated using a PCR strategy. Two PCR fragments were amplified from a

pPinPoint-14-3-3A construct using gene specific primers G216Sf (5'-GGACTCCC TCTCCGAGGAATC-3') and G216Sr (5'-GATTCCTCGGAGAGGGAGTCC-3'), containing a *Bsl*I restriction site, and vector specific primers SP6 (5'-CGTGAC GCGGTGCAGGGCG-3') and PinPoint (5'-ATTTAGGTGACACTATAG-3'). The reactions were carried out for 30 cycles with an annealing temperature of 55°C and *Tfl* proofreading polymerase (Promega, Madison, WI, USA) according to manufacturer's instructions. The 5'fragment (SP6 and G216Sf) was digested with *Not*I (Gibco BRL, Gaithersburg, MD, USA), the 3'fragment was digested with *Sty*I (New England Biolabs, Beverly, MA, USA) and the original pPinPoint-14-3-3A construct was digested with both mentioned enzymes. Digestions were carried out O/N. Enzymes were inactivated for 20 min at 65°C and removed with phenol/chloroform. DNA was precipitated using 100% EtOH and dissolved in H_2O. Both PCR fragments were further digested by *Bsl*I (New England Biolabs) for 6 hrs and cleaned as above. Fragments of all digests were separated on a 1% agarose TAE gel, bands corresponding to the expected sizes were excised and DNA was extracted using a gel extraction kit (Qiagen, Valencia, CA, USA). The fragments were ligated, desalted and transformed into JM109. Positive colonies were checked by sequencing, the 14-3-3A^{G216S} fragment was transferred into the pRSETC vector and used for production as the WT constructs. Transformed BL21 (DE3) pLysS cells (Invitrogen) were grown O/N at 37°C in 2 x YT, 1 % glucose and 50 µg/ml ampicillin until the OD_{600} was 0.8 - 0.9. Expression was induced by replacing the medium by 2 x YT, 1 mM isopropyl-β-D-thiogalactoside (AppliChem, Darmstadt, Germany) and 50 µg/ml ampicillin and incubation at 28°C for 4 h. Cells were pelleted at 5000 ***g*** for 15 min and dissolved in 30 ml 20 mM Hepes/KOH pH 7.5, 0.5 M NaCl, 1 mM PMSF and l0 mM imidazol (Merck, Darmstadt, Germany) at 4°C. Once dissolved, 1 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and 4 µg/ml deoxyribonuclease 1 (Sigma-Aldrich) were added and incubated for 30 min. The cell lysate was centrifuged for 30 min at 100 000 ***g*** and supernatant filtered through a 0.45 µM filter (Schleicher & Schuell, Dassel, Germany). The filtrate was applied to a Ni^{2+} charged HiTrap Chelating HP column (Amersham Pharmacia, Uppsala, Sweden) and subjected to a 10 mM to 500 mM imidazol gradient. The 14-3-3 peak was pooled, desalted to 1 mM Hepes/KOH pH 7.5 using a HiPrep 26/10 desalting column (Amersham) and concentrated by freeze drying. Quantity

and quality of the recombinant proteins were checked using the Bradford protein assay (BioRad, Hercules, CA, USA), Coomassie stained SDS-PAGE gel (Fig. 8).

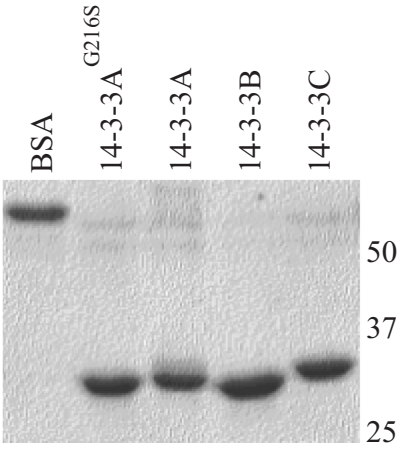

Figure 8. Purified recombinant 14-3-3 isoforms. Coomassie stained SDS-PAGE gel showing purity, quantity and quality of histidine labelled 14-3-3 isoforms A^{G216S}, A, B and C (2 µg) and as a control BSA (2 µg).

Far-UV circular dichroism

Far-UV CD spectra of recombinant 14-3-3 proteins (100 µg/ml in 5 mM Na-phosphate pH 7.5) were obtained using a Jasco J-715 spectropolarimeter (Jasco, Easton, MD, USA), equipped with a Jasco PTC-348 WI to maintain the temperature at 20°C. Spectra were acquired from 260 to 190 nm with a continuous scanning mode, a scanning speed of 20 nm/min, 0.5 nm interval, a band width of 1 nm and 1 sec response times using a quartz cuvette (type 110-QS, Hellma, Muellheim, Germany) with a 1 mm light path. All spectra are the average of 5 independent scans and buffer blank (5 mM Na-phosphate pH 7.5) spectra, obtained at identical conditions, were substracted to compensate for solvent contribution. The analysis of far-UV CD spectra in secondary content was performed using CDNN version 2.1 software (Institut für Biotechnologie, Martin Luther Universität Halle Wittenberg, Germany).

NR extraction

Barley (*Hordeum vulgare* cv. Alexis, Josef Breun Saatzucht, Herzogenaurach, Germany) plants were grown in soil for 7 days, fed with 100 mM KNO_3 24 hrs before harvesting and placed in the dark 30 min before harvesting. Leaves (5 g) were ground in liquid nitrogen, followed by the addition of 10 ml extraction buffer (100 mM Hepes/KOH pH 7.5, 10 % (v/v) glycerol, 20 mM NaF, 10 mM EDTA, 20 µM FAD, 5 µM $NaMoO_4$, 0.1 % (v/v) Triton X-100, 6 mM DTT, 5 µM cantharidin, 2 mM PMSF, complete protease inhibitor (3 tablets/500 ml) and 1 % (w/v) PVPP). The suspension was filtered through 4 layers of Miracloth (Calbiochem, La Jolla, CA, USA) and the filtrate was centrifuged at 100 000 ***g*** for 1 hr at 4°C. The supernatant was filtered through a 0.45 µM filter (Schleicher & Schuell, Dassel, Germany), the filtrate was applied to a HiTrap Q anion exchange column (Amersham) and subjected to a 0 mM to 500 mM NaCl gradient in extraction buffer (without PVPP). NR activity was localised as described in Figure 1 and pooled for further experiments.

Determining the 14-3-3 protein content

HiTrap Q protein (5 µl) fractions were separated on 12% SDS-PAGE. The separated proteins and control recombinant 14-3-3 proteins (0.5 pmol) were transferred to Immun-Blot PVDF Membrane (BioRad, Hercules, CA, USA). The membrane was probed with isoform-specific Anti-14-3-3 antibodies as described previously (Testerink et al., 1999).

NR assay

NR activity was tested by adding 50 µl of NR extract to 500 µl of either EDTA assay buffer (50 mM Hepes/KOH pH 7.5, 5 mM EDTA, 10 mM KNO_3 and 0.5 mM NADH), Mg Assay buffer (50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 10 mM KNO_3 and 0.5 mM NADH) or Ca assay buffer (50 mM Hepes/KOH pH 7.5, 5 mM $CaCl_2$, 10 mM KNO_3 and 0.5 mM NADH). The mixture was incubated at 30°C for 30 min and the reaction was stopped by the addition of 50 µl 1 M ZnAc. After centrifuging at 13 000 ***g*** for 2 min, 500

µl of the supernatant was mixed with 500 µl of 0.01% NAP and 0.5% Sulfanilamide in 0.75 N HCl. The absorbance of this mixture was measured at 540 nm.

Surface plasmon resonance

Surface plasmon resonance experiments were performed with a Biacore 2000 (Biacore, Uppsala, Sweden). A streptavidin coated sensor chip (Biacore) was activated with 1 M NaCl, 50 mM NaOH, flowpath 3 was coated at 5 µl/min with 1000 response units of NR-P peptide (Biotin-CPGLKRSTS^{P}TPFMN) (Ansynth, Roosendaal, The Netherlands) in immobilization buffer (10 mM KAc, pH 6 with HAc). Measurements were done in running buffer (20 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$/$CaCl_2$, 25 mM NaCl and 15 mM imidazol), at 25°C using a flow rate of 10 µl/min. An association step of 3 min, with a 14-3-3 isoform diluted in running buffer, was followed by a dissociation step of 3 min in running buffer. The chip was regenerated between runs using 0.5 % SDS (w/v), 50 mM NaCl pH 6.8. The data was processed using BIAevaluation 3.1. (Biacore).

Acknowledgements

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Chapter 4

A small-scale purification method

for the identification of 14-3-3 target proteins

In collaboration with Dave Speijer, Peter J. Schoonheim, Ilja Roobeek,
Daniel da Costa Pereira, Jos N.M. Mol and Albertus H. de Boer

Summary

Members of the family of 14-3-3 proteins function in the regulation of a wide variety of cellular processes through interaction with key target molecules. In recent years, hundreds of, mainly mammalian, potential target molecules have been identified using affinity purification. In order to enable comprehensive identification of 14-3-3 interacting proteins from barley, a small-scale affinity purification approach is developed using the extensively studied nitrate reductase (NR) model system. NR activity was effectively separated from a partially purified extract with the use of 14-3-3C coated magnetic beads and specific elution with competing peptides, resulted in a sufficient quantity of protein to allow positive identification of the purified NR by MALDI-TOF mass spectrometry. Applying this method to more crude barley leaf and root extracts, resulted in the purification of several 14-3-3 interacting proteins, of which neutral invertase could be positively identified. This result was further confirmed by the presence of neutral invertase activity in the eluted fraction and the significant inhibition of neutral invertase activity upon addition of 14-3-3C.

Introduction

In order for cells to maintain homeostasis and adequately respond to environmental changes, control over protein activity is crucial. In recent years, the highly conserved family of 14-3-3 proteins was shown to play a key role in the regulation of the activity of many proteins. The existence of 14-3-3 proteins in plants was first described in 1992 (De Vetten et al., 1992; Hirsch et al., 1992; Lu et al., 1992). Within a few years 14-3-3 proteins were shown to function as part of the receptor for the phytotoxin fusicoccin (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994) and identified as the inhibitor protein of nitrate reductase (NR) (Bachmann et al., 1995; Bachmann et al., 1996b; Moorhead et al., 1996). To date over 100 targets for 14-3-3 proteins have been identified. Through these target proteins, 14-3-3 proteins affect processes like ion homeostasis (Bunney et al., 2002; De Boer, 2002), assembling and targeting of protein complexes (Rajan et al., 2002), metabolism (Comparot et al., 2003), signal transduction (Camoni et al., 1998), prevention of apoptosis (Yoshida et al., 2005) and cell cycling (Van Hemert et al., 2001). The number of 14-3-3 target proteins and the processes affected by 14-3-3 proteins is still expanding as is reflected by the steady increase in publications concerning 14-3-3 proteins. With few exceptions 14-3-3 proteins act through binding to two distinct phosphorylated motifs, R/KxxS^{p}/T^{p}xP (Mode-1) and R/KxxxS^{p}/T^{p}xP (Mode-2), in target proteins (Muslin et al., 1996; Yaffe et al., 1997; Rittinger et al., 1999). Searching the predicted protein products of the *Arabidopsis* genome sequence for potential 14-3-3 interacting proteins based on these two motifs shows that 40% of all proteins contain the 14-3-3 recognition sequence (Sehnke et al., 2002a). This indicates that many more proteins and processes might be regulated by 14-3-3 proteins.

To gain a better understanding of the control 14-3-3 proteins exert on cellular processes, identification of additional 14-3-3 interacting proteins is crucial. As previously discussed by Huber et al. (2002), the use of common methods to identify protein binding partners, like yeast two-hybrid, is limited due to a lack of phosphorylation of 14-3-3 interacting proteins. Alternatively, 14-3-3 affinity chromatography was successfully applied to identify several novel 14-3-3 interacting proteins from cauliflower (Moorhead et al., 1999). More recently, a similar method resulted in the identification of hundreds of 14-3-3

interacting partners in mammalian cells (Rubio et al., 2004; Benzinger et al., 2005). However, this method requires large quantities of starting material. For example, Moorhead et al. (1999) used 10 mg of recombinant 14-3-3 proteins to purify target proteins from 1 kg of cauliflower extract.

We have used the NR model system to set up an alternative small-scale 14-3-3 affinity purification method. The regulation of NR is one of the best-studied mechanisms of 14-3-3 action in plants. NR represents the first step of N-fixation in plant cells by catalysing the reduction of nitrate to nitrite. Further reduction of nitrite to ammonia depends on electrons donated by photosynthesis. To prevent accumulation of the toxic nitrite, dark induced phosphorylation of the hinge 1 region of NR (Douglas et al., 1995; Su et al., 1996) enables 14-3-3 proteins to interact and consequently inactivate NR (Kaiser and Huber, 2001; MacKintosh and Meek, 2001; Comparot et al., 2003). Using the NR model system allows us to follow the efficiency and optimise conditions in purifying 14-3-3 interacting proteins.

Here we study a 14-3-3 affinity purification approach in which recombinant 14-3-3 proteins are immobilized to streptavidin coated beads. The advantage of this method over the previously used affinity chromatography (Moorhead et al., 1999) is the simplified purification method (Smith, 2005) and the potential use in automated affinity purification platforms. We show that 14-3-3 interacting proteins can be purified using a relative small amount of starting material as compared to the previous used method (Moorhead et al., 1999) by following NR activity during the purification process. Applying this affinity purification method to more crude extracts resulted in the identification of the cytosolic neutral invertase as a target protein for 14-3-3 regulation. Further, parallel affinity purifications using the three characterised barley 14-3-3 isoforms were performed to identify potential 14-3-3 isoform specific interacting partners.

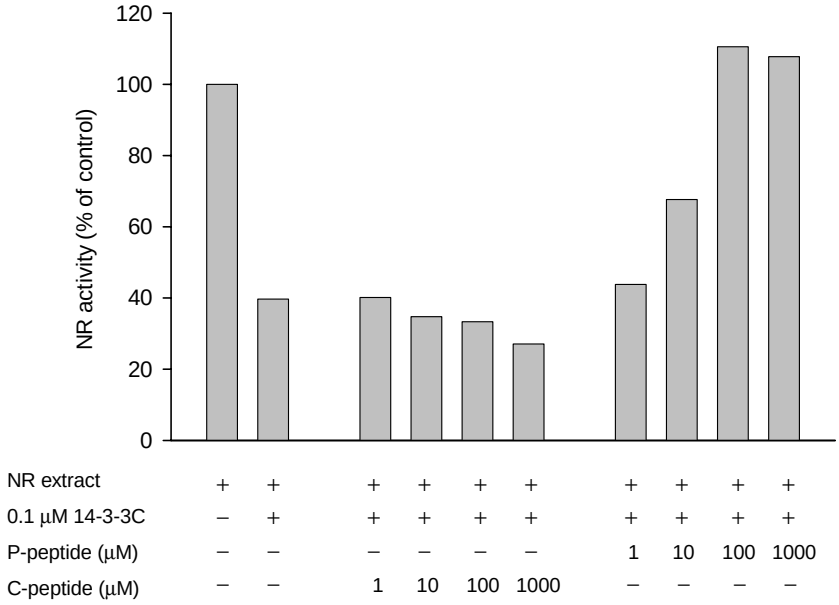

Figure 1: Effect of (phospho-)peptides on 14-3-3C inhibition of NR. NR activity from 7 days old barley leaves was partially purified and the activity of 50 µl extract was tested in 0.5 ml Mg assay buffer. Addition of 0.1 µM 14-3-3C results in a reduction of NR activity of 60%. The inhibitory action of 14-3-3C was titrated with increasing amounts of a peptide containing the 14-3-3 interaction motif (RRSRS$^{(P)}$AP) with either phosphorylated serine (P-peptide) or non-phosphorylated serine (C-peptide).

Results

The inhibition of NR by 14-3-3 interaction is one of the best studied examples of 14-3-3 regulation in plants. Therefore, we used the NR model system to set up a 14-3-3 affinity purification strategy. NR activity was isolated from dark harvested barley leaves and partially purified to separate NR activity from native 14-3-3 proteins as previously described (Sinnige et al., 2005a). The affinity purification strategy is based on the inhibitory effect, and thus binding, of recombinant 14-3-3 proteins upon incubation with the NR extract (Fig. 1) (Bachmann et al., 1996a; Moorhead et al., 1996; Moorhead et al., 1999; Sinnige et al., 2005a). Multiple 14-3-3 isoforms have been identified in barley (Brandt et al., 1992; Testerink et al., 1999), we chose to use 14-3-3C since we previously showed that this isoform has the highest affinity for barley NR (Sinnige et al., 2005a). For

specific elution of the isolated 14-3-3 interacting proteins, different concentrations of a phosphopeptide (P-peptide), representing the 14-3-3 binding motif (previously designated KCO-P (Sinnige et al., 2005b)), and the non-phosphorylated equivalent (C-peptide) were tested for their ability to compete with NR for 14-3-3 proteins (Fig. 1). Clearly, concentrations of 0.1 to 1 mM of the P-peptide are suitable for specific elution of 14-3-3 interacting proteins. The C-peptide can be used as control since even a concentration of 1 mM does not reduce the action of 14-3-3C (Fig. 1).

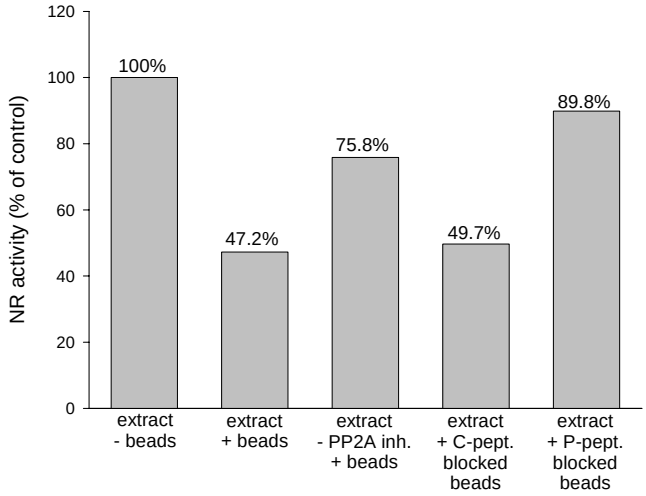

Figure 2: 14-3-3C coated beads remove NR activity from extract. NR extract was isolated in the presence and absence of the phosphatase inhibitors NaF and cantharidin. 100 µl NR extract (with 10 mM $MgCl_2$) was incubated for 10 min with 14-3-3C coated beads (100 µl) in the absence and presence of (phospho-)peptide. After removal of the beads from the extract, the supernatant was assayed for NR activity in EDTA assay buffer. Extraction of NR activity by 14-3-3C coated beads was efficiently prevented by addition of P-peptide but not by C-peptide.

Coupling the biotinylated 14-3-3C proteins to streptavidin coated metal beads enables the separation of 14-3-3 interacting proteins from the extract using a magnetic stand. The ability of the 14-3-3 proteins to interact with target proteins is unaffected after coupling to the beads and P-peptide still efficiently prevents binding of 14-3-3C to interacting proteins (Fig. 2). The 14-3-3C coated beads were subjected to consecutive incubations with fresh NR extract to obtain an indication of the NR binding capacity (Fig. 3). The percentage of NR activity removed from the extract decreases from 53% in the first to 35% in the last

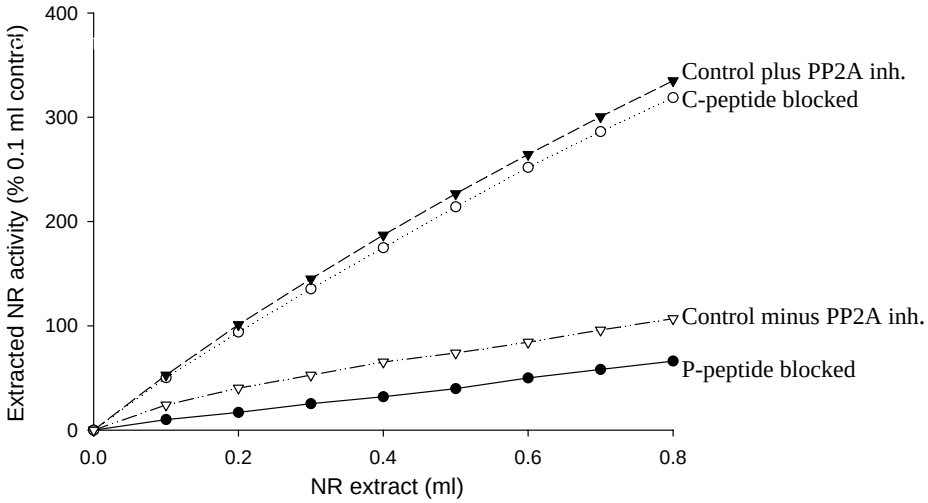

Figure 3: Cumulative extractions of NR activity showing the NR binding capacity of 14-3-3C coated beads. NR extract was isolated as described in Fig. 2. Successive NR extract fractions (100 µl) were incubated for 10 min with 14-3-3C coated beads in the presence and absence of (phospho-)peptides. The cumulative percentage of NR activity removed during successive incubations (expressed in % of control) is plotted against the cumulative volume (ml).

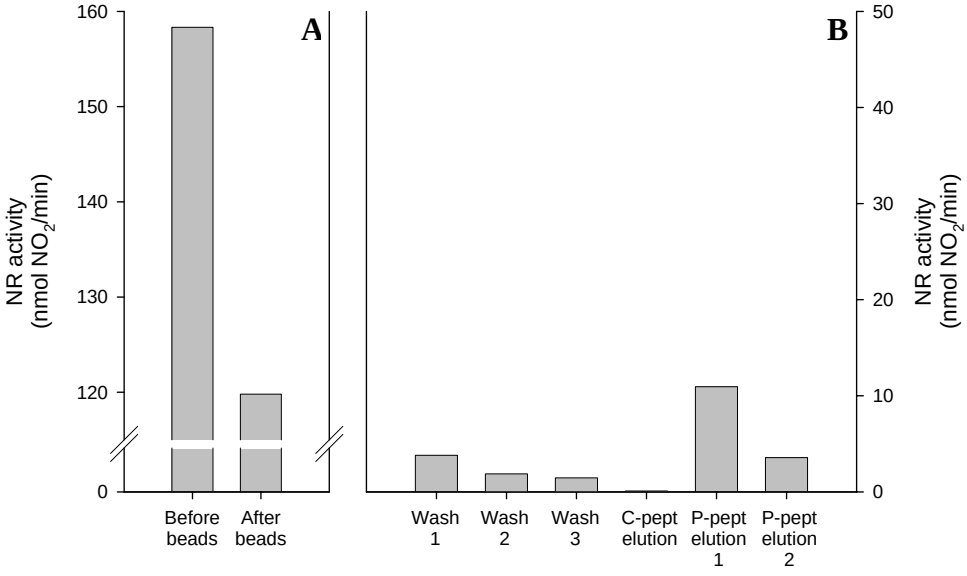

Figure 4: NR activity during consecutive steps in affinity purification of NR. (A) Total NR activity present in 4 ml extract before and after incubation with 14-3-3C coated beads and (B) the NR activity in wash and elution steps show specific elution of NR activity upon P-peptide elution.

incubation. Based on the observation that there was still a significant amount of NR activity extracted during the last incubation, we used 4 ml of NR extract for the affinity purification experiment to saturate the 14-3-3C coated beads with NR. Further, in line with the previously reported necessity of NR to be phosphorylated to allow 14-3-3 interaction (Kaiser and Huber, 2001; MacKintosh and Meek, 2001; Comparot et al., 2003), affinity purification from an extract that was isolated in the absence of phosphatase inhibitors NaF and cantharidin, resulted in a reduction of the removed NR activity (Fig. 2 & 3).

To further analyze the affinity purification method of 14-3-3 interacting proteins, we followed NR activity during the complete procedure. The 14-3-3C coated magnetic beads were consecutively incubated with 4 times 1 ml NR extract. After removal of the extract from the beads, the total NR activity in the extract was reduced by 38.5 nmol NO_2/min (Fig. 4A). Based on the activity of purified NR from corn seedling (Sigma-Aldrich), this corresponds roughly with 1 to 4 µg of extracted NR protein. Three consecutive washes of the beads in 1 ml wash buffer resulted in the loss of 3.8, 1.9 and 1.5 nmol NO_2/min of NR activity, respectively (Fig. 4B). Note that the presence of NR cofactors FAD and $NaMoO_4$ during these washes was crucial to obtain any NR activity in these and later eluting fractions (data not shown). For the specific elution of NR from the 14-3-3C coated beads, they were first incubated with 50 µl 1 mM C-peptide followed by 50 µl 1 mM P-peptide. The supernatant of these elutions contained 0.10 and 10.94 nmol NO_2/min NR activity, respectively (Fig. 4B). This shows that the affinity purification method allows purification and specific elution of 14-3-3 interacting proteins, although about 50% of the NR activity removed from the original NR extract is still unaccounted for. To determine whether all NR activity was released from the beads, a second P-peptide elution was applied to the beads. This released another 3.6 nmol NO_2/min NR activity (Fig. 4B), which shows that, although 1 mM P-peptide was sufficient to fully block NR inhibition in Figure 1, a significant part of the NR was still bound to 14-3-3 after the first P-peptide elution.

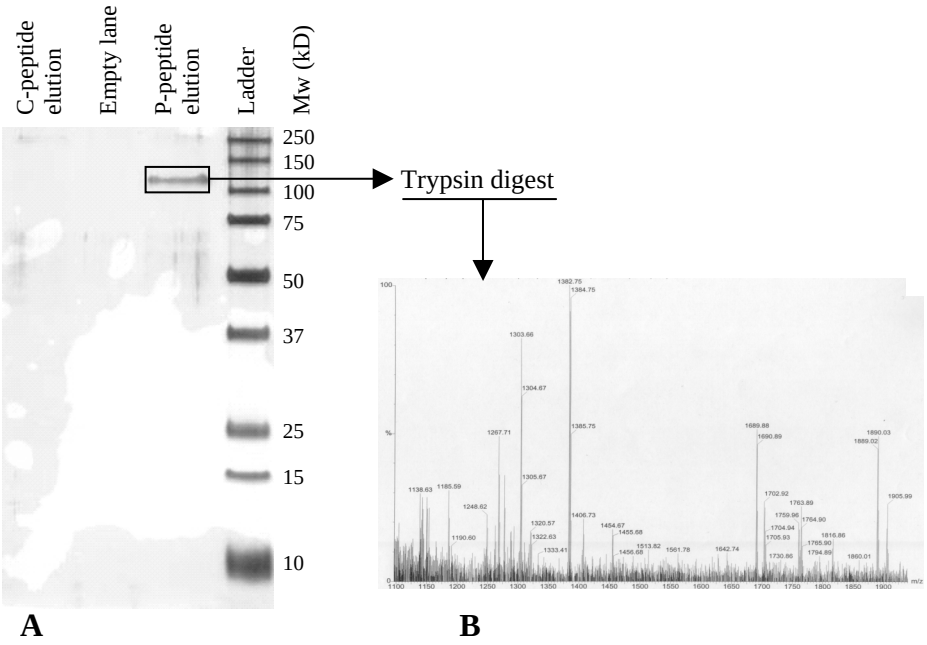

Figure 5: Identification of purified proteins. Silver stained SDS-PAGE showing proteins eluted from 14-3-3C coated beads with KCO-C and KCO-P (A). A 110 kD band was cut from gel and trypsin digested. Resulting peptides were subjected to MALDI-TOF (B) for identification.

Matching peptides MW	Residue		Sequence
	Start	End	
1137.6506	640	651	(K)VAGAPIALSSPR
1148.6230	842	850	(K)VWYVIDQVK
1184.5938	756	766	(K)GPLGHVEYTGR
1286.6442	38	49	(R)GCGFPSLISPPR
1381.7241	858	869	(K)FSVGFVTEDILR
1453.6586	448	459	(R)AWDQTHNTQPEK
1626.8981	673	688	(R)FALPSSDQVLGLPVGK
1688.8522	147	161	(R)GDWATWTVEVTGLVR
1701.8985	109	123	(R)LTGKHPLNCEPPLAR
1814.8799	73	88	(R)EAYGSHLQLEVEPSTR
1887.9930	124	139	(R)LMHHGFITPAPLHYVR
1941.9544	92	108	(R)DEGTADAWIERNPSLIR
1941.9585	308	323	(R)RIVVTTAESDNYYHFK
2140.0762	704	722	(R)AYTPTSMVDEIGQFELLVK
2481.1859	485	507	(K)GEIGLVFEHPTQPGNQTGGWMAR

Table 1: NR matching peptides identified by mass spectrometry

For the identification of 14-3-3 interacting proteins the C-peptide elution and the P-peptide elution were subjected to SDS-PAGE and visualised by silver staining (Fig. 5A). Differential bands allow discrimination between 14-3-3 interacting proteins and nonspecific interacting proteins and in line with the observed NR activity in these elutions, the silver stained gel shows a clear protein band with a molecular mass slightly over 100 kD in the P-peptide elution lane where no visible protein band could be detected in the lane of the C-peptide elution. This differential band was excised, digested by trypsin and MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) mass spectra of the resulting peptides (Fig. 5B) were compared to protein databases to identify the 14-3-3 interacting protein. The best fit of peptide masses with a protein (table 1), with a MOWSE score of $2.8e^{14}$ and 22.5% of the protein covered with matching peptides, was indeed barley NR (GenBank P27969).

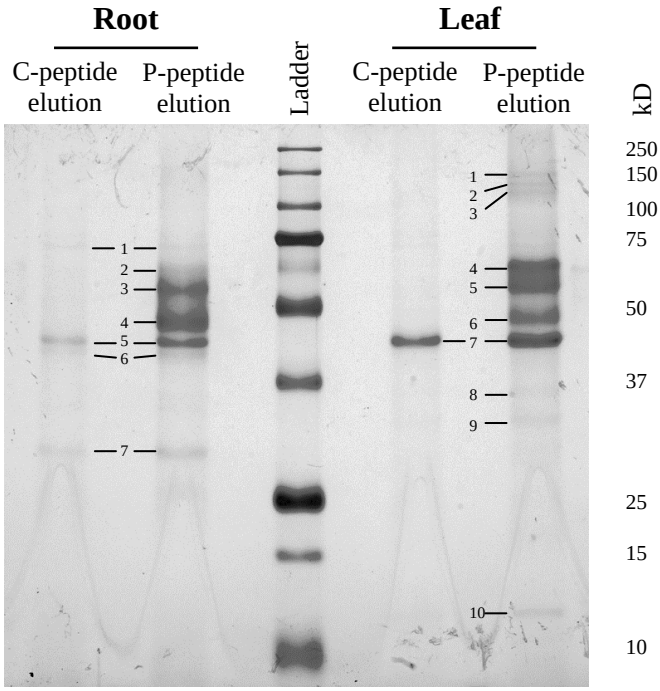

Figure 6: Silver stained SDS-PAGE gel of affinity purified 14-3-3C interacting proteins from crude barley root and leaf extract. Seven distinct protein bands are present in the P-peptide elution after affinity purification in root extract of which three bands (2, 3 and 4) are differential bands compared to the C-peptide elution. Affinity purification in barley leaf extract resulted in ten distinct protein bands in the P-peptide elution of which band 7 is also found in the C-peptide elution. Differential bands were excised and subjected to MALDI-TOF analysis.

For the identification of other, potentially new, 14-3-3 interacting proteins we applied the affinity purification method to protein extracts of barley root and leaf tissue. Peptide elutions were subjected to SDS-PAGE and visualised by silver staining. The P-peptide elution of affinity purification in root tissue showed seven distinct protein bands of which three were differential bands compared to the C-peptide elution (Fig. 6). From leaf tissue, nine differential protein bands were isolated, including three bands (4-6) with the same molecular mass as the three differential bands found using root tissue and a band (3) with the molecular mass of NR (Fig. 6). All differential bands were excised, trypsin digested and subjected to MALDI-TOF analysis. Unfortunately, the quantity of the less intense bands isolated from leaf tissue (1-3, 8-10) was not sufficient to obtain workable spectra. The MALDI mass spectra of the remaining differential bands, isolated from root (2-4) and leaf (4-6) tissue, showed a surprisingly high similarity. Since comparing the mass spectra to protein databases did not result in the identification of these proteins, two peptides (ions 858.5 MH^+ and 1107.5 MH^+), present in all these spectra, were further analysed by tandem mass spectrometry. The resulting sequences (AIDLAEAR and LGEGAMPASFK, respectively) identified these proteins, based on homology with rice (GenBank CAE03040) and Arabidopsis (GenBank O80556) protein, as putative neutral invertases.

Invertases (EC 3.2.1.26) are a group of enzymes that irreversibly hydrolyse sucrose into glucose and fructose (Tymowska-Lalanne and Kreis, 1998a). The cytosolic neutral invertases, represented by different isoenzymes (Tymowska-Lalanne and Kreis, 1998a), have a molecular mass between 58 and 66 kDa (Rosario and Santisopasri, 1977; Vorster and Botha, 1999; Bosch et al., 2004) and function as an octamer (Lee and Sturm, 1996) with an optimum between pH 6.8 and 8.0 (Roitsch and Gonzalez, 2004). In order to validate the presence of neutral invertases in the P-peptide elution, we determined the invertase activity in the extract and the P-peptide elution (Fig. 7a). Clearly, a 7 to 8-fold increase in invertase activity was found in the P-peptide elution as compared to the activity in the extract. To further assess the effect of 14-3-3C, extract was assayed for invertase activity in the presence and absence of 0.3 μM 14-3-3C (Fig. 7b). Addition of 14-3-3C resulted in a 30% inhibition of invertase activity.

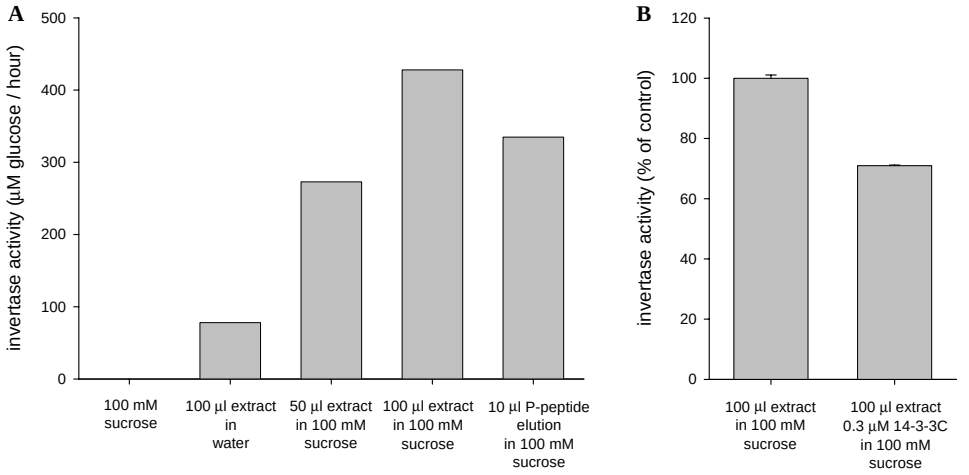

Figure 7: Barley neutral invertase interacts with and is inhibited by 14-3-3C. In vertase activity was measured using Nelson's test after incubation for 30 min at 30ºC in 100 mM sucrose at pH 7.5. (A) Neutral invertase activity in 10 µl P-peptide elution after affinity purification from barley leaf extract as compared to the neutral invertase activity in 50 and 100 µl of leaf extract. Invertase activity measurements in 100 mM sucrose and 100 µl leaf extract in water were performed as controls. (B) Inhibition of invertase activity upon addition of 0.3 µM 14-3-3C protein (n = 3, ±SD).

Multiple 14-3-3 isoforms are found in all tested eukaryotic tissues. To address the question whether molecular diversity in 14-3-3 isoforms leads to functional diversity (Roberts, 2000; Roberts and de Bruxelles, 2002; Paul et al., 2005; Sinnige et al., 2005a), we used all three described barley 14-3-3 isoforms (Testerink et al., 1999) in parallel to affinity purify target proteins. P-peptide elutions of these affinity purifications in both barley root and leaf extract were subjected to SDS-PAGE and visualised by silver staining (Fig. 8). Several putative interacting proteins are visible for all 14-3-3 isoforms. Interestingly, mostly proteins of similar size are present in the P-peptide elution of all three 14-3-3 isoforms, although the relative intensity of the bands sometimes varies which possibly reflects differences in affinity. However, some bands (indicated by triangles) appear to be differential. The identity of these purified proteins remains unclear, as they were not subjected to MALDI-TOF analysis. The molecular mass of the most prominent bands corresponds with the putative neutral invertases (Fig. 6).

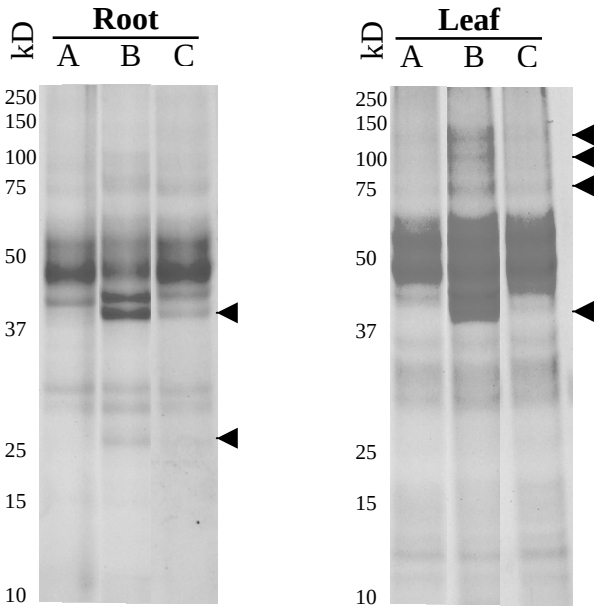

Figure 8: Silver stained SDS-PAGE gels loaded with 10 µl of P-peptide elution show affinity purified proteins from crude barley root and leaf extract using three 14-3-3 isoforms (A, B and C). Affinity purifications with different 14-3-3 isoforms were performed simultaniously using the same barley leaf/root extract. Using different 14-3-3 isoforms resulted in few differential protein bands (triangles) in the P-peptide elutions.

Discussion

Identification of 14-3-3 interacting proteins will provide better insight in the control 14-3-3 proteins exert on cellular processes. In recent years, affinity chromatography has been successfully used to identify hundreds of new, mainly mammalian, 14-3-3 interacting proteins (Moorhead et al., 1999; Cotelle et al., 2000; Milne et al., 2002; Rubio et al., 2004; Benzinger et al., 2005). In the present work, we studied the efficiency of a similar affinity purification strategy using streptavidin coated metal beads as a matrix. The potential advantage of these beads over the previously used affinity chromatography lies in simplified purification method (Smith, 2005). The beads are easy to handle and no centrifugation or columns are required, which also reduces the time consumption. Further, elution of interacting proteins can be performed in small volumes, so there is no need to concentrate the obtained sample for further analysis. Finally, the development of platforms able to use these beads for automated affinity purification allows high

throughput screening of 14-3-3 interacting proteins using for example multiple 14-3-3 isoforms under varying physiological conditions and/or in different tissues.

Important for successful isolation of 14-3-3 interacting proteins, biotinylation of the 14-3-3 molecules using pPinPoint and subsequent coupling it to the strepavidin coated metal beads does not affect its binding capabilities as shown by the ability to reduce NR activity (Fig 1 & 2). The most powerful part of the purification method, as previously recognised and used for affinity chromatography of 14-3-3 interacting proteins (Moorhead et al., 1999; Milne et al., 2002), is the specific elution of 14-3-3 interacting proteins by competing with the, 14-3-3 binding motif carrying, synthetic peptides. In previous work we determined the affinity of 14-3-3C for both P-peptide and NR to be 82 nM (Sinnige et al., 2005b) and 0.5 nM (Sinnige et al., 2005a) respectively. The relatively high affinity of 14-3-3C for NR as compared to P-peptide, explains the excess of P-peptide necessary for efficient elution of NR (Fig. 1). Discrimination between the elution of 14-3-3 interacting proteins and non-specifically bound proteins is accomplished by pre-elution using the non-phosphorylated variant (C-peptide), which generally has a lower affinity for 14-3-3 proteins (Moorhead et al., 1996; Muslin et al., 1996; Yaffe et al., 1997) and is therefore unable to compete with 14-3-3 interacting proteins like NR (Fig. 1 & 4). Subsequent elution with P-peptide releases 14-3-3 interacting proteins and screening for differential proteins between these elutions allows specific discrimination for 14-3-3 interacting proteins.

By following the activity of one of the best studied models for 14-3-3 action in plants, NR, during the purification process, we demonstrate that this affinity purification method for 14-3-3 interacting proteins provides a sensitive way to specifically isolate NR from a partially purified NR extract in quantities sufficient enough to allow positive identification (Fig. 4 & 5). Applying the method to more crude extracts of root and leaf tissue resulted in the purification of several putative 14-3-3 interacting proteins (Fig. 6). Unfortunately, several of these proteins could not be identified because the quantity of the isolated protein was too low to allow positive identification. The specifically eluted NR activity represents approximately 30% of the extracted activity (Fig. 4). Some of the NR activity was lost during the washing steps, most likely due to the formation of a new equilibrium.

Another part remained coupled to 14-3-3 during elution as shown by a second P-peptide elution. Therefore, combining successive P-peptide elutions would increase the success rate concerning the positive identification of low abundant 14-3-3 interacting proteins. Alternatively, up scaling the method would have the same effect. The amount of starting material used in this study (recombinant 14-3-3 proteins, plant tissue and peptide) as well as the final yield of the purification, correspond to approximately 0.5% of the materials used and final yield as reported for the affinity purification method used by Moorhead et al. (1999). The rest of the lost NR activity may be the result of loss of enzyme activity over time, especially since all purification steps were performed at room temperature.

Surprisingly, the proteins that were present in sufficient quantity in the purification from crude root and leaf extract were all identified as neutral invertases. The cytosolic neutral invertases irreversibly hydrolyse sucrose into glucose and fructose (Tymowska-Lalanne and Kreis, 1998b) and therewith represent, besides sucrose-phosphate synthase (Toroser et al., 1998; Moorhead et al., 1999) and trehalose-6-phosphate synthase (Moorhead et al., 1999), the third 14-3-3 regulated protein in the plant sugar metabolism. Interestingly, a group of proteins described as LIM17-related proteins in the previous 14-3-3 affinity purification by Moorhead et al. (1999) are similar in size and share a peptide sequence (LGEGAMPASFK) with these neutral invertases. The interaction of 14-3-3 with neutral invertase was confirmed by both the presence of neutral invertase activity in the P-peptide elution and the reducing effect of 14-3-3C on the invertase activity in crude leaf extract (Fig. 7). As neutral invertases are represented by multiple isoenzymes (Tymowska-Lalanne and Kreis, 1998b) and function as an octamer (Lee and Sturm, 1996), it is unclear whether all three isolated neutral invertase masses (and if not, which) interact with 14-3-3 proteins. One should take into account that the interaction between 14-3-3 and any protein purified using this or a similar method should be confirmed since it could form complex with 14-3-3 interacting proteins and be purified as such.

As in recent work on 14-3-3 isoforms clear differences in target affinity and functionality are observed between these isoforms (Bachmann et al., 1996a; Kurz et al., 2000; Rosenquist et al., 2000; Emi et al., 2001; Alsterfjord et al., 2004; Paul et al., 2005; Sinnige et al., 2005a; Sinnige et al., 2005b), we performed an affinity purification experiment

using the same extract on all three thus far described barley 14-3-3 isoforms (Fig. 8). In contrast to these observed functional differences, the majority of the isolated protein bands is present in the affinity purification of all three used 14-3-3 isoforms. This might be explained by the relatively high concentration of 14-3-3 during the affinity purification. As shown in reports where the effect of different concentrations of multiple 14-3-3 isoforms were tested on target proteins (Bachmann et al., 1996a; Sinnige et al., 2005a; Sinnige et al., 2005b), high concentrations of 14-3-3 can mask differences in affinity since they will eventually bind the target protein. Differences in intensity of some of the purified putative 14-3-3 target proteins between the three affinity purifications therefore also potentially reflect differences in affinity between the different 14-3-3 isoforms. Finally, a few proteins bands are not present in all purifications indicating that these proteins do not interact with all 14-3-3 isoforms and therefore indicate functional specificity.

In the end, the eluted NR and newly identified neutral invertases were proven sufficient in quantity to allow positive identification as 14-3-3 interacting proteins using MALDI analysis (Fig. 5). In general, the method allows specific purification of 14-3-3 interacting proteins using a fraction (0.5%) of the starting material as compared to a previously described method (Moorhead et al., 1999). However, the obtained yield represented the same fraction (0.5%) of the yield obtained previously (Moorhead et al., 1999). As a result, the quantity of several purified 14-3-3 interacting proteins was not sufficient to allow positive identification. This might be solved by the use of more starting material. Alternatively, the affinity-purified proteins could be separated using liquid chromatography (LC), as retrieving the proteins from silver stained SDS-PAGE gels results in a major loss of quantity. The method used in this work has some important advantages in terms of a reduction in handling steps and time consumption. Further, the method allows the purification of target proteins from a relative small amount of starting material and enables comparing different tissues/growth conditions in performing parralel purifications. Finally, concerning the affordability of the method, a recent report describes a method to efficiently regenerate the streptavidin coated magnetic beads (Holmberg et al., 2005).

Experimental procedures

Plant material and protein extraction

Barley (*Hordeum vulgare* cv. Alexis, Josef Breun Saatzucht, Herzogenaurach, Germany) plants were grown in soil for 7 days, fed with 100 mM KNO_3 24 hrs before harvesting and placed in the dark 30 min before harvesting. Barley leaf and/or root tissues (5 gr) were ground in liquid nitrogen using a mortar and pestle, followed by the addition of 10 ml ice-cold extraction buffer (100 mM Hepes/KOH pH 7.5, 10 % (v/v) glycerol, 20 mM NaF, 10 mM EDTA, 20 µM FAD, 5 µM $NaMoO_4$, 0.1 % (v/v) Triton X-100, 6 mM DTT, 5 µM Cantharidin, 2 mM PMSF, Complete protease inhibitor (3 tablets/500 ml) and 1 % (w/v) PVPP). The suspension was filtered through 4 layers of Miracloth (Calbiochem, La Jolla, CA, USA) and the filtrate was centrifuged at 100 000 *g* for 1 hr at 4°C. The supernatant was filtered through a 0.45 µM filter (Schleicher & Schuell, Dassel, Germany), and the filtrate was used for affinity purification experiments. For the purification of NR, the supernatant was applied to a HiTrap Q anion exchange column (Amersham Pharmacia, Uppsala, Sweden) and subjected to a 0 mM to 500 mM NaCl gradient in extraction buffer (without PVPP). NR activity was localised with an NR assay and pooled for further experiments.

NR assay

NR activity was tested by adding 50 µl of NR extract to 500 µl of Mg assay buffer (50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 10 mM KNO_3 and 0.5 mM NADH) or EDTA assay buffer (50 mM Hepes/KOH pH 7.5, 5 mM EDTA, 10 mM KNO_3 and 0.5 mM NADH). The mixture was incubated at 30°C for 30 min and the reaction was stopped by the addition of 50 µl 1 M ZnAc. After centrifuging at 13 000 *g* for 2 min, 500 µl of the supernatant was mixed with 500 µl of 0.01% NAP and 0.5% Sulfanilamide in 0.75 N HCl. The absorbance of this mixture was measured at 540 nm.

Invertase assay

Invertase activity was tested by adding the extract to 100 mM sucrose (1 ml total volume) at pH 7.5 and incubating for 30 min at 30ºC. Reduced sugar compounds were quantified with Nelson's test as previously described (Nelson, 1944). 1 ml Nelson's reagent (12.5 ml reagent A (12.5 gr Na_2CO_3 (anhydrous), 12.5 gr potassium sodium tartrate, 10 gr $NaHCO_3$ and 100 gr Na_2SO_4 dissolved in 500 ml H_2O) mixed with 0.5 ml reagent B (7.5 gr $CuSO_4.5H_2O$ and 1 drop H_2SO_4 in 50 ml H_2O)) was added to 1 ml invertase reaction, mixed and heated in a boiling water bath for 20 min. The reaction was cooled on ice and 1 ml arsenomolybdate reagent (25 gr $(NH_4)6.Mo_7O_{24}.4H_2O$, 21 ml H_2SO_4 (concentrated) in 450 ml H_2O mixed with 3 gr $Na_2HAsO_4.7H_2O$ in 25 ml H_2O, stored at 37ºC) was added. The mixture was incubated for 5 min and shaken occasionally before 7 ml H_2O was added. The absorbance of this mixture was measured at 540 nm.

Expression of recombinant biotinylated 14-3-3 isoforms

Recombinant biotinylated barley 14-3-3 isoforms were cloned and expressed as previously described (Van den Wijngaard et al., 2001). Briefly, a BamH1 fragment was cloned into pPinPoint (Promega, Madison, WI, USA). JM109 cells containing the construct were grown at 37°C in 500 mL LB containing 50 µg/ml ampicillin until OD_{600} was between 0.6 and 0.8. Isopropyl-β-D-thiogalactoside (IPTG) was added to a concentration of 0.1 mM and cells were incubated at 28°C for 4 h to induce expression. Cells were pelleted at 5000 *g* for 15 min and dissolved in 30 ml 20 mM Hepes/KOH pH 7.5, 0.5 M NaCl, 1 mM PMSF and l0 mM imidazol (Merck, Darmstadt, Germany) at 4°C. Once dissolved, 1 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and 4 µg/ml deoxyribonuclease 1 (Sigma-Aldrich) were added and incubated for 30 min. The cellysate was centrifuged for 30 min at 100 000 *g* and supernatant filtered through a 0.45 uM filter (Schleicher & Schuell). The filtrate was applied to a HiTrap Q anion exchange column (Amersham Pharmacia) and subjected to a 10 mM to 500 mM NaCl gradient in 5 mM Hepes/KOH pH 7.5. 14-3-3 proteins were localized using spot-blotting and specific antibodies. Positive fractions were pooled and stored at -20°C.

Affinity purification

Recombinant biotinylated barley 14-3-3 proteins (2 nmol) were incubated for 1 hr with 1 mg avidin coated metal beads (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. Beads were incubated twice for 10 min with 1 ml 20 mM Hepes/KOH pH 7.5, 1 mg/ml biotin to block free avidin sites and twice for 10 min with 1 ml 20 mM Hepes/KOH pH 7.5 to wash away unbound molecules. In some cases, 14-3-3 proteins were blocked for 30 min with 1 mM KCO-P peptide (Biotin-GAKRFRRSRS(PO_3)APRSE) or KCO-C peptide (Biotin-GAKRFRRSRSAPRSE) (Ansynth, Roosendaal, The Netherlands) in 20 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$. Beads were incubated on a rotator with NR extract plus 5 mM $MgCl_2$ for 10 min. Beads were washed 3 times 10 min in 1 ml wash buffer (100 mM Hepes/KOH pH 7.5, 20 mM NaF, 10 mM EDTA, 20 µM FAD, 5 µM $NaMoO_4$, 0.1 % (v/v) Triton X-100, 6 mM DTT, 5 µM Cantharidin, 2 mM PMSF, Complete protease inhibitor (3 tablets/500 ml) and 10 mM $MgCl_2$). Proteins bound to 14-3-3 were selectively eluted by successively incubating for 30 min in 50 µl C-peptide elution buffer (1 mM KCO-C peptide in wash buffer) and 50 µl P-peptide elution buffer (1 mM KCO-P peptide in wash buffer).

Protein identification by mass spectrometry

Eluted proteins were separated on a 12 % SDS-PAGE gel and stained by silver staining (Nesterenko et al., 1994). Protein bands were cut and gel slices were washed for 10 min in 100 µl 100 mM NH_4HCO_3, dehydrated for 30 min in 50% (v/v) acetonitrile and vacuum dried. Gel pieces were incubated in 100 µl of a solution containing 10 mM DTT and 100 mM NH_4HCO_3 for 1 hr at 56ºC. The supernatant was removed and replaced by 100 µl 55 mM iodoacetamide and 100 mM NH_4HCO_3 for 45 min in the dark at room temperature to alkylate SH-groups. After removal of the supernatant, gel pieces were washed for 10 min with 100 µl 100 mM NH_4HCO_3, dehydrated by adding 100 µl 100% acetonitrile, rehydrated in 100 µl 100 mM NH_4HCO_3, dehydrated in 100% acetonitrile and vacuum dried. Gel pieces were swollen on ice in 50 µl digestion buffer containing 12.5 ng/µl trypsin (sequencing grade; Boehringer, Mannheim, Germany), 125 µM HCl and 50 mM

NH_4HCO_3. After 45 min, the supernatant was replaced with 30 µl of the same buffer without trypsin and incubated O/N at 37ºC. The supernatant was collected and peptides were eluted for 45 min at room temperature with 150 µl 20 mM NH_4HCO_3, followed by 3 successive elutions with 150 µl 100% acetonitrile. Elutions were pooled and dried O/N in a vacuum centrifuge. Peptides were dissolved in 6 µl 60 % acetonitrile and 1% (v/v) formic acid. The peptide solution was mixed 1:1 with 52 mM α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in 50% (v/v) ethanol / 48% acetonitrile / 2% (v/v) trifluoroacetic acid and 1 mM ammonium acetate, 0.5 µl of this mixture was spotted on a M@LDI target plate (Micromass, Wythenshawe, UK) and dried at room temperature. MALDI-TOF MS spectra of the peptides were obtained using a Micromass M@LDI R and Mass Lynx ProteinProbe software (Micromass) was used to identify proteins by searching the Non-Redundant Protein Database (National Center for Biotechnology Information, USA) and the International Protein Index (European Bioinformatics Institute, UK). During the search the following parameters were applied for confident protein identification: no restrictions on species or p*I*, maximum molecular weight of 110 kDa, 0 or 1 missed cleavages, maximum mass tolerance of 50 ppm (unless in a clear linear progression from the internal calibration mass reference 2163.06), with carboxyamidomethylation of cysteines as fixed modification and methionine oxidations as variable modification. The match details for the protein with the highest MOWSE score were manually checked to ensure proper identification (adapted from (Sprenger et al., 2004)).

Acknowledgements

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Chapter 5

Identification and characterisation of two novel barley 14-3-3 isoforms

Parts of this chapter have been submitted for publication in collaboration with
Peter J. Schoonheim, Jose A. Casaretto, Tom D. Bunney,
Ralph S. Quatrano and Albertus H. de Boer

Summary

Proteins of the highly conserved 14-3-3 family have distinct functions as regulators of primary metabolism and ion homeostasis. Recent studies strongly indicate functional isoform specificity between the different 14-3-3 isoforms. The biological function of a given 14-3-3 isoform seems to be dependent on a combination of the capability and the availability of that isoform. In this work, we aim to improve the insight in the availability of 14-3-3 isoforms in barley. Thus far, three 14-3-3 isoforms have been described for barley, whereas recent reports suggest that eight 14-3-3 isoforms are present in the related rice. This suggests the presence of additional 14-3-3 isoforms in barley. Using the strong sequence conservation between 14-3-3 isoforms in a PCR based screening, we identified two novel barley 14-3-3 isoforms, 14-3-3D and 14-3-3E. The relative expression of all five 14-3-3 isoforms in several barley tissues is determined and provide an indication for the availability of isoforms in regulating several processes. Further, in the light of recent indications that 14-3-3 proteins might serve a function in ABA induced seed dormancy, the expression of the 14-3-3 isoforms and their response to ABA treatment in embryonic barley roots (radicles) was tested.

Introduction

Initially discovered as brain specific proteins (Moore and Perez, 1967), 14-3-3 proteins have now emerged as a group of abundant regulatory proteins. The list of known 14-3-3 targets nowadays contains hundreds of proteins and is growing rapidly (Milne et al., 2002; Pozuelo Rubio et al., 2004; Benzinger et al., 2005; Satoh et al., 2005). The regulatory functions ascribed to 14-3-3 proteins are diverse: they include controlling metabolic enzymes (Bachmann et al., 1996b; Toroser et al., 1998; Cotelle et al., 2000), ion transport activity (Bunney et al., 2002; De Boer, 2002), proteins kinases (Aitken et al., 1992; Irie et al., 1994), gene transcription (De Vetten et al., 1992; Lu et al., 1992) and protein assembly (O'Kelly et al., 2002; Rajan et al., 2002).

All tested eukaryotic organisms contain multiple 14-3-3 isoforms, ranging from two in *Saccharomyces cerevisiae* to twelve expressed isoforms in *Arabidopsis* (Rosenquist et al., 2000; Rosenquist et al., 2001). In plants, there seems to be a divergence in the number of isoforms between dicotyledons and monocotyledons. Where twelve functional 14-3-3 isoforms were described for the dicot Arabidopsis (Rosenquist et al., 2001) and similar numbers are found for tomato and tobacco (Roberts, 2003), only eight 14-3-3 isoforms were identified by analysing the rice genome (Jin et al., 2005). In barley, which was among the first plants in which 14-3-3 proteins were discovered (Brandt et al., 1992), only three 14-3-3 isoforms (A, B and C) are described in literature thus far (Brandt et al., 1992; Testerink et al., 1999).

One major issue concerning 14-3-3 proteins is the function of this large number of isoforms. The amino acid sequence of the 14-3-3 proteins is very well conserved, both between the different isoforms within an organism as well as between evolutionary divergent species like yeast, human and *Arabidopsis*. Along with other indications, this led to the suggestion that these different isoforms are functionally redundant in their ability to act on target proteins and multiple 14-3-3 isoforms function in providing each cell/cellular compartment with the desired quantity of 14-3-3 proteins (Palmgren et al., 1998; Roberts, 2000; Zuk et al., 2005). However, evidence is accumulating that small variations between the 14-3-3 isoforms lead to discrimination between target proteins

(Sinnige et al., 2005a; Wilker et al., 2005) and are responsible for the observed functional differences between 14-3-3 isoforms (Bachmann et al., 1996a; Rosenquist et al., 2000; Emi et al., 2001; Bornke, 2005; Sinnige et al., 2005b).

Besides functional capability, the availability of 14-3-3 isoforms is an important parameter in determining a biological role for a given 14-3-3. Clear differential expression patterns of 14-3-3 isoforms have been observed in, for example, barley embryos (Testerink et al., 1999; Maraschin et al., 2003a; Maraschin et al., 2003b), *Arabidopsis* root tissue (Sehnke et al., 2002b) and tomato root and fruit tissues (Roberts and de Bruxelles, 2002). In analysing the regulation of barley NR, 14-3-3B and 14-3-3C were shown to efficiently inhibit NR activity, whereas 14-3-3A was ineffective (Sinnige et al., 2005a). However, 14-3-3C was hardly present in the relevant tissue, leaving 14-3-3B as the main candidate to regulate barley NR *in vivo* (Sinnige et al., 2005a).

In the present study we describe the isolation of two novel barley 14-3-3 isoforms, Hv14-3-3D and Hv14-3-3E. For a better insight in the availability of the five different barley isoforms, we determined the relative expression of these isoforms in different barley tissues.

Results

Identification of novel barley 14-3-3 genes

Three barley 14-3-3 isoforms have been described in literature thus far (Brandt et al., 1992; Testerink et al., 1999). However, a recent search in the protein database of the sequenced rice, which is closely related to barley, revealed four novel rice 14-3-3 isoforms, in addition to the four already known rice 14-3-3 isoforms (Jin et al., 2005). This suggests the presence of additional barley 14-3-3 isoforms, available to regulate target proteins. A PCR based strategy, using the relative high sequence conservation between 14-3-3 proteins, yielded two novel barley 14-3-3 genes (Fig. 1). These two new 14-3-3 isoforms, denoted Hv14-3-3D and Hv14-3-3E, have a similarity of 75% and both have > 70% similarity with the other three already known 14-3-3 isoforms.

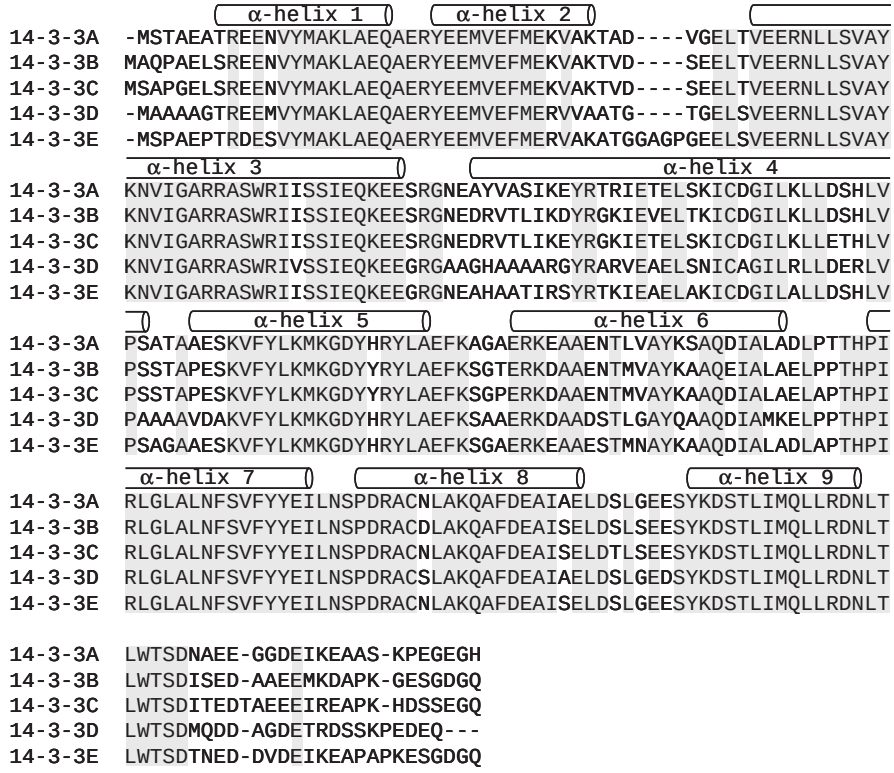

Figure 1. Sequence conservation among barley 14-3-3 isoforms. Amino acid sequence alignment of the five known barley 14-3-3 isoforms. Identical amino acids are marked in gray and predicted α-helices (1-9) are indicated above the sequence. The newly identified 14-3-3D and 14-3-3E have a similarity of 75% and both have > 70% similarity with the three already known 14-3-3 isoforms.

A notable difference between Hv14-3-3E and the other isoforms is found in the loop between helix 2 and 3, where Hv14-3-3E contains four extra amino acids. This isoform seems to be specific to monocots, as a similar insertion of four amino acids is present in the rice GF14-d and the wheat TaWIN1, but not in any *Arabidopsis*, tomato or tobacco 14-3-3 isoform. Phylogenetic analysis of the barley 14-3-3 isoforms together with in the GenBank and TIGR databases available 14-3-3 isoforms from rice and wheat (table 1), indeed confirms an evolutionary relationship between these three isoforms (Fig. 2).

Further, the second novel barley isoform, *Hv14-3-3D*, appears to be an orthologue of the rice *GF14-f* and the wheat *TaWIN2*. In contrast to Jin et al. (2005), our analysis of the GenBank and TIGR databases revealed only seven rice 14-3-3 isoforms (table 1), indicating that at least two barley 14-3-3 isoforms remain to be identified.

Organism	Name	Accession number	Database
Hordeum vulgare	Hv14-3-3A	X62388	GenBank
	Hv14-3-3B	X93170	GenBank
	Hv14-3-3C	Y14200	GenBank
	Hv14-3-3D	DQ295785	GenBank
	Hv14-3-3E	DQ295786	GenBank
Oryza sativa	GF14-a (S94)	D16140	GenBank
	GF14-b	U65956	GenBank
	GF14-c	U65957	GenBank
	GF14-d	U65958	GenBank
	GF14-e	TC249902	TIGR
	GF14-f	TC250944	TIGR
	GF14-g	TC267894	TIGR
Triticum aestivum	Ta14-3-3A	TC233195	TIGR
	Ta14-3-3B	TC263093	TIGR
	Ta14-3-3C	TC246776	TIGR
	TaWIN1	AB042193	GenBank
	TaWIN2	AB042194	GenBank

Table 1 List of 14-3-3 isoforms from barley, wheat and rice available in the GenBank and TIGR databases that were used in the present study.

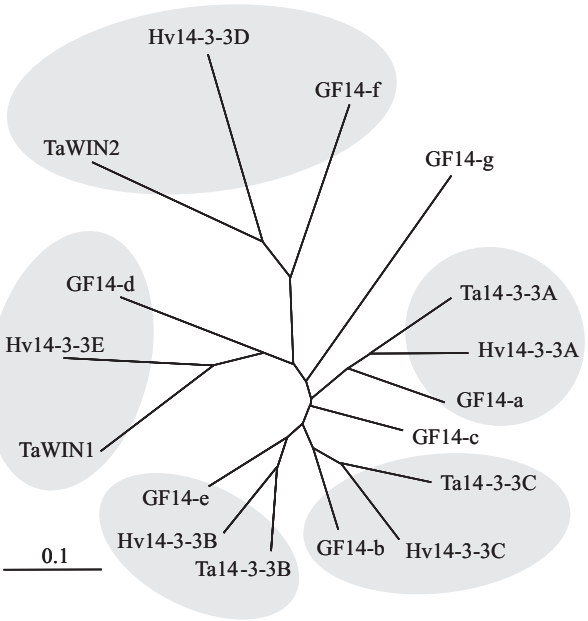

Figure 2. A non-rooted phylogenetic tree showing the relationship between the known 14-3-3 isoforms from barley, wheat and rice (table 1). Five clusters of orthologous genes can be identified. Genes orthologous to *GF14-c* and *GF14-g* remain to be identified in barley and wheat. Alignment of cDNA's was produced using AlignX (Vector NTI) and the plot was produced by Treeview.

Differential expression of barley 14-3-3 isoforms

To obtain insight in the biological role that the different 14-3-3 isoforms fulfill in barley, it is crucial to know the availability of 14-3-3 isoforms in the different tissues of the plant. Therefore, the relative expression of all five 14-3-3 isoforms was tested in different tissues from several developmental stages (Fig. 3). Although expression of all isoforms was found in all examined tissues, major variations in expression levels were found for different isoforms. The expression of *14-3-3A* is relatively high in all tissues, where hardly any transcript of *14-3-3D* was detected. *14-3-3B* seems to be ubiquitously expressed, although the relative expression is slightly lower in radicles and the root-tip.

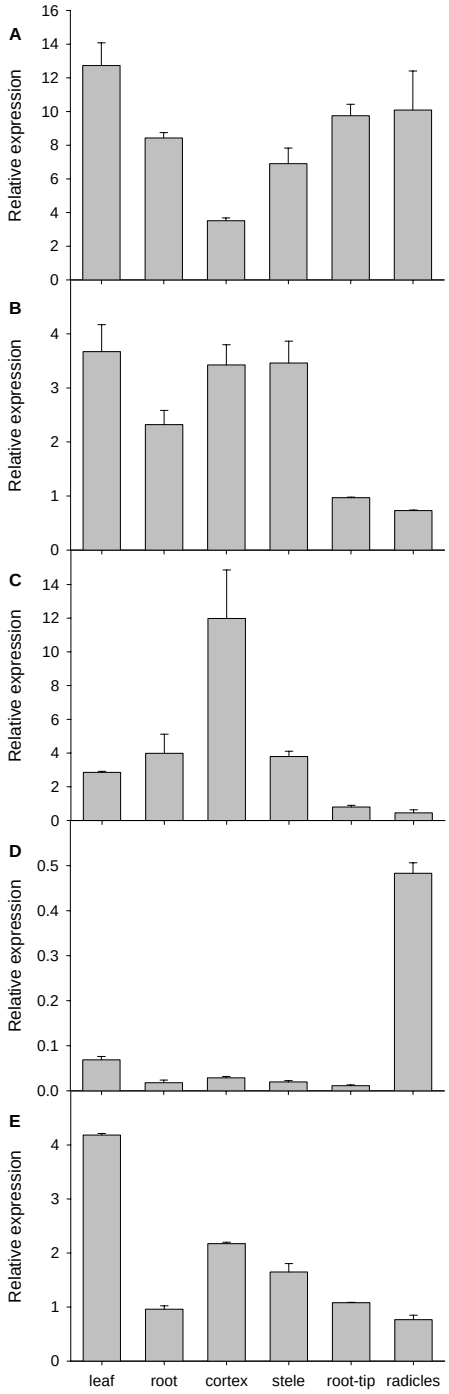

Figure 3. Relative expression of the barley 14-3-3 isoforms in different tissues of barley. Figures A to E correspond to 14-3-3 isoforms A, B, C, D and E, respectively. Leaf and root tissue was harvested from 1-week-old plants; root-tips, cortical and stelar tissue was isolated from 3-week-old secondary roots; radicles were isolated 14 h after imbibition of barley seeds. Expression was determined using quantitative RT-PCR and normalised to the expression of actin (*n* = 3; mean ± S.D.).

The expression profile of *14-3-3C* seems opposite to that observed for *14-3-3A* and is mainly found in cortical tissue of mature roots. The observed expression of the three previously described isoforms (A, B and C) is in agreement with reported immunolocalizations of these isoforms in barley embryos, where 14-3-3A and 14-3-3B were found in all tissues, whereas the presence of 14-3-3C was limited to specific tissues (Testerink et al., 1999). Concerning the newly identified 14-3-3 isoforms, considerable *14-3-3E* expression is found in all tested tissues with a slightly higher expression in leaf tissue. Interestingly, expression of *14-3-3D*, which is hardly expressed as compared to the other isoforms, is 10 to 50 times higher in radicles than in the other tested tissues. This observation and the potential role of 14-3-3 proteins in abscisic acid (ABA) signalling (Van den Wijngaard et al., 2005), led us to study the expression of the 14-3-3 isoforms during barley embryonic root growth in more detail.

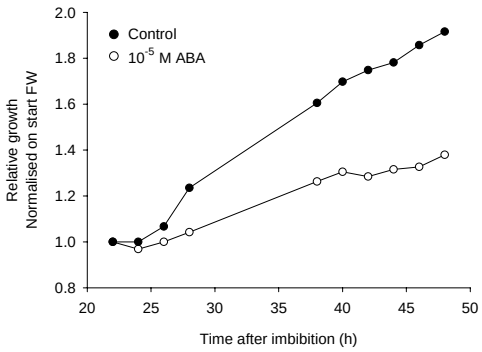

Figure 4: Effect of ABA on radicle growth. Relative growth (weight increase as compared to t = 22) of isolated radicles is followed in time. Radicles were isolated 22 h after imbibition of barley seeds and grown in the absence (●) and presence of 10^{-5} M ABA (○).

14-3-3 isoforms in the primary root of barley embryos and their response to ABA

The first evidence that 14-3-3 proteins may function in ABA induced growth arrest of barley radicles was provided by a study of ion channel regulation in radicle protoplast (Van den Wijngaard et al., 2005). Moreover, 14-3-3 proteins were shown to interact with VP1 (Schultz et al., 1998), a transcription factor that together with the transcription factor ABI5 plays an important role in regulating expression of ABA inducible genes (Casaretto and Ho, 2003). These studies point to the possibility that the 14-3-3 proteins function both in ABA signalling and on the downstream effectors of the ABA pathway, ion channels and pumps. Therefore, the expression of the five 14-3-3 isoforms in barley radicles was

tested in the absence and presence of ABA (Fig. 5). With the exception of t = 14, radicles were isolated 20 hours after imbibition, just prior to the emergence of the coleorhiza. The growth of the control and ABA treated radicles (Fig. 4) was similar to the previous reported data (Van den Wijngaard et al., 2005). The relative expression data show that four of the five 14-3-3 isoforms respond to ABA treatment. Levels of *14-3-3A* expression are not significantly affected after addition of ABA, whereas ABA induces a transient up-regulation of *14-3-3B* and *14-3-3C*, peaking at six hours after addition of ABA. Further, ABA induces a strong and sustained increase in expression of the two novel 14-3-3 genes, *14-3-3D* and *14-3-3E*. These results provide further evidence for a function of some or more of the 14-3-3 isoforms in ABA mediated seed dormancy.

Discussion

In the last decade, the family of 14-3-3 proteins emerged as major regulators of various cellular processes. The presence of multiple 14-3-3 isoforms raised questions concerning the role of these different isoforms. In recent years, evidence is accumulating that some 14-3-3 isoforms have a higher affinity for certain target proteins, whereas other isoforms prefer different targets, indicating functional isoform specificity (Bachmann et al., 1996a; Rosenquist et al., 2000; Bornke, 2005; Sinnige et al., 2005b). This discrimination between target proteins is likely to be the result of small variations in the structure of the different isoforms (Sinnige et al., 2005a; Wilker et al., 2005). Besides the functional capability of 14-3-3 isoforms to regulate target proteins, availability of isoforms is an obvious requirement. Indeed, differential expression of 14-3-3 isoforms is observed in several tissues (Daugherty et al., 1996; Testerink et al., 1999; Roberts and de Bruxelles, 2002; Sehnke et al., 2002a; Maraschin et al., 2003b). In regulating barley NR activity, both 14-3-3B and 14-3-3C were shown very effective (Sinnige et al., 2005a). However, 14-3-3C was barely present in the relevant tissue, leaving 14-3-3B as the only isoform able to function in NR inhibition (Sinnige et al., 2005a). Thus, to obtain insight in the biological role that the different 14-3-3 isoforms fulfill, it is crucial to analyse the capability and availability of the 14-3-3 isoforms. In this work we focus on the availability of 14-3-3 isoforms in barley.

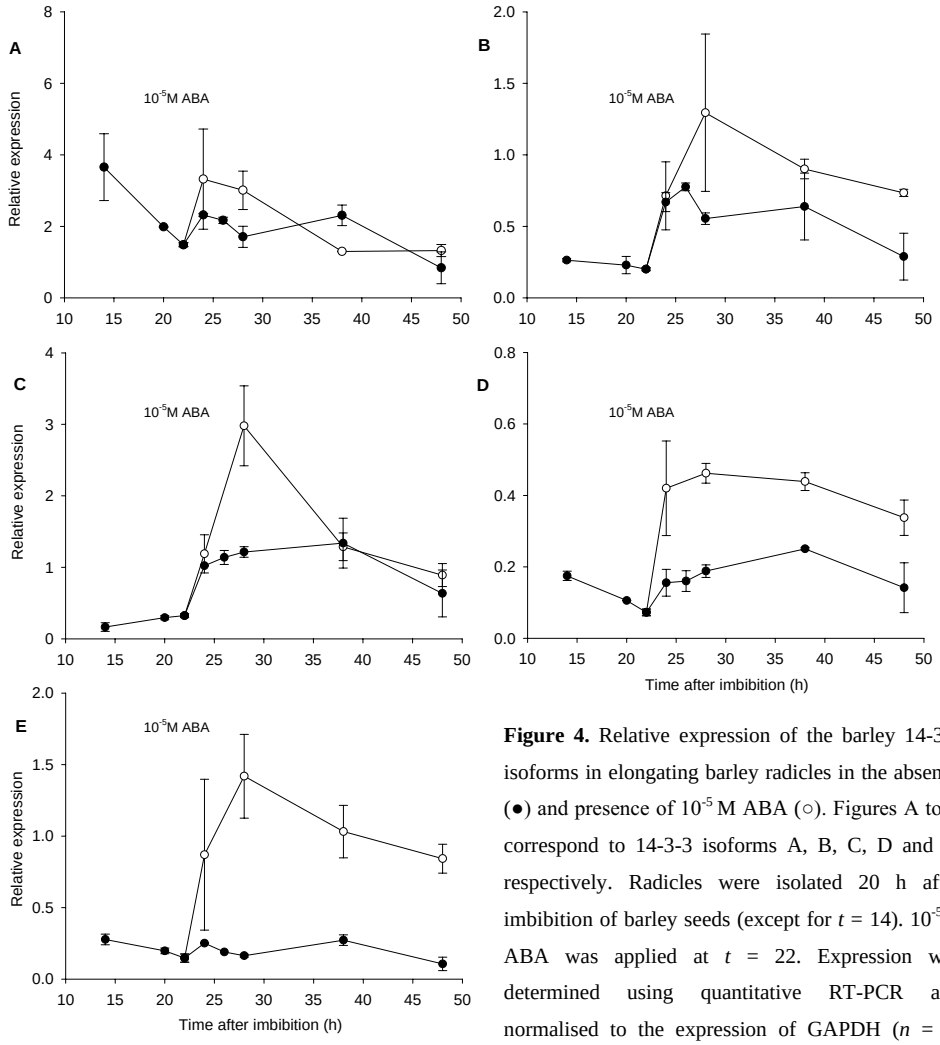

Figure 4. Relative expression of the barley 14-3-3 isoforms in elongating barley radicles in the absence (●) and presence of 10^{-5} M ABA (○). Figures A to E correspond to 14-3-3 isoforms A, B, C, D and E, respectively. Radicles were isolated 20 h after imbibition of barley seeds (except for t = 14). 10^{-5}M ABA was applied at t = 22. Expression was determined using quantitative RT-PCR and normalised to the expression of GAPDH (n = 3; mean ± S.D.).

Until the genome of barley is sequenced and analysed, it is difficult to determine the exact number of 14-3-3 isoforms. Thus far, three barley isoforms have been described in literature (Brandt et al., 1992; Testerink et al., 1999). Recent analysis of rice databases revealed eight different rice 14-3-3 isoforms (Jin et al., 2005). Although our search in the TIGR and GenBank database only resulted in seven rice 14-3-3 isoforms (Table 1), this indicated that there might be additional barley 14-3-3 isoforms. In this study we identified

the genes of two novel barley 14-3-3 isoforms, *14-3-3D* and *14-3-3E* (Fig. 1). Phylogenetic analysis shows that these isoforms are the barley orthologues of already known rice (*GF14-f* and *GF14-d*, respectively) and wheat (*TaWIN2* and *TaWIN1*, respectively) isoforms. An interesting characteristic of 14-3-3E is the presence of four additional residues between helix 2 and 3 (Fig. 1). These additional residues are also found in GF14-d and TaWIN1, but not present in the known 14-3-3 isoforms of Arabidopsis, tomato, tobacco and potato. The structural and functional consequences (e.g. dimer formation and target recognition) of these additional residues remain unclear, but are certainly worth looking into since they seem to be conserved in monocotyledons. Further, the phylogenetic analysis of barley, wheat and rice 14-3-3 isoforms shows five clusters of orthologous 14-3-3 isoforms plus two single rice isoforms, *GF14-c* and *GF14-g* (Fig. 2). A search in the barley and wheat EST databases (TIGR), using these two rice isoforms as a template, did not result in the identification of the corresponding genes, whereas *14-3-3D* and *14-3-3E* were present in the barley EST database (data not shown).

In order to obtain an indication of the availability of the different 14-3-3 isoforms in various barley tissues, we analysed the relative expression of the 14-3-3 isoforms. In line with previous studies (Daugherty et al., 1996; Testerink et al., 1999; Roberts and de Bruxelles, 2002; Sehnke et al., 2002a; Maraschin et al., 2003b), we observed clear differences in expression level of the 14-3-3 genes throughout the plant (Fig. 3). Striking is the low abundance of *14-3-3D* transcripts in comparison with *14-3-3A*. Further, some isoforms appear predominantly expressed in specific tissues. The expression of *14-3-3D* in radicles is 10 to 50 times higher as compared to the other tested tissues and *14-3-3C* transcripts are significantly more abundant in the root cortical tissue. These observations are in line with the reported tissue specific and differential expression of barley 14-3-3 isoforms during several stages of barley embryogenesis (Testerink et al., 1999; Maraschin et al., 2003a). The expression measured in this study should be considered a first indication for the availability of 14-3-3 isoforms. A more detailed analysis, using for example immunolocalization or promoter-GUS studies, is necessary to determine the availability of 14-3-3 isoforms in specific cell types and/or subcellular compartments.

We previously used elongating embryonic roots (radicles) from germinating barley seeds to study the effect of 14-3-3 and ABA on ion channel activity. These results indicated a function for 14-3-3 proteins in ABA induced seed dormancy (Van den Wijngaard et al., 2005). Further evidence for this role of 14-3-3 proteins is provided by the observed effect of ABA on the expression of 14-3-3 isoforms: a transient increase in *14-3-3B* and *C* transcripts combined with the sustained up-regulation of *14-3-3D* and *E* (Fig. 5). This increase in expression suggests a requirement for these 14-3-3 isoforms to prevent further germination. This theory is supported by measurements of 14-3-3 protein levels in this tissue, where 14-3-3C, D and E are no longer present at $t = 46$ in the control situation but upon ABA treatment the protein level is maintained (Schoonheim et al, unpublished data). Interestingly, 14-3-3 proteins are also shown to interact with VP1 (Schultz et al., 1998). This VP1 is a transcription factor and functions, together with ABI5, in regulating expression of ABA inducible genes (Casaretto and Ho, 2003). The identification of several 14-3-3 isoforms as ABA inducible genes (Fig. 5) provides the possibility that 14-3-3 proteins (as component of the VP1/ABI5 complex) are involved in regulating expression of (other) 14-3-3 isoforms.

Experimental procedures

Plant material

Barley (*Hordeum vulgare* cv. Alexis, Josef Breun Saatzucht, Herzogenaurach, Germany) plants were grown in hydroponics (1/4 strength Long Ashton) in a controlled environmental growth chamber with a 14 h light / 10 h dark cycle at 20 and 18ºC, respectively. Barley radicles were isolated and maintained as described previously (Van den Wijngaard et al., 2005).

RNA isolation and expression analysis

For expression experiments, total RNA from barley tissues was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was treated with DNase and first-strand cDNA was produced using SuperScript reverse transcriptase (Invitrogen) and oligo(dT) primers.

Quantitative RT-PCR (DNA Engine Opticon, MJ Research, Inc., Waltham, MA, USA) was carried out with SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) using primers: 14-3-3A fw (5'-GTAGGATGGTGCTATGCGAAGC-3'), rev (5'-ACTAAGAAGCGACGATGTCCACA-3'), 14-3-3B fw (5'-GAGGCTCCAATTTGTGTTGTGATA-3'), rev (5'-ACAGGAAGGTTCAACAAGAGGCAA-3'), 14-3-3C fw (5'-GTTGCATGCAATGTGGTCTGGAT-3'), rev (5'-AACCTTCCCCGGCAACATCA-3'), 14-3-3D fw (5'-GTGGACCTCTGATATGCAGGATG-3'), rev (5'-AAGGTTAAGAGAGAAAGAGCGCAGT-3'), 14-3-3E fw (5'-ATGAGATAAAGGAAGCCCCAGC-3'), rev (5'-GAACTAGAACCAAGACCATCGGC-3'), GAPDH fw (5'-CCTTCCGTGTTCCCACTGTTGA-3'), rev (5'-GGTTTCCCTCAGACTCCTCCTTGA-3'), Actin fw (5'-GTATGGAAACATCGTGCTCAGTGG-3') and rev (5'-CTTGATCTTCATGCTGCTCGGA-3'). All kits were used according to the manufacturers protocol.

Identification of Hv14-3-3D and Hv14-3-3E isoforms

To identify new barley 14-3-3 isoforms a degenerated primer (5'-RGAYTC5AC5YT5ATHATG-3', U.I.B. coding and where a 5 denotes an inosine) was designed against a homologues part in the 3' end of most of the known plant 14-3-3's. PCR was performed using this degenerated primer as forward and the 3' RACE primer as reverse. The amplified fragments were cloned into a pGEM-T (promega) vector. Colonies were screened using filter lifting, and filters were hybridised with a radiolabeled probe directed against the conserved region of the *Hv14-3-3C* cDNA. Positive clones were sequenced using theT7 promoter and Bigdye sequencing kit (Applied Biosystem). Performing 5' RACE the full-length cDNA sequence was identified. The full cDNA sequence was cloned in frame into the pinpoint Xa (promega) vector.

Acknowledgements

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Summary

Summary

Cellular processes like the cell cycle, ion transport, gene expression and enzyme activity are tightly controlled, e.g. in order to prevent tumor formation, maintain homeostasis and adequately respond to environmental changes (e.g. light intensity, osmotic potential and pathogen attack). Understanding the mechanisms by which organisms/cells regulate protein activity and function, and thereby cellular processes, potentially allows manipulation of these control mechanisms in for example treating diseases/infections or increasing crop yield. The work described in this thesis was aimed to obtain a better understanding of the role and function of a specific class of regulators, the family of 14-3-3 proteins.

As described in Chapter 1, the family of 14-3-3 proteins has emerged as important regulators of many proteins that are involved in a wide variety of cellular processes. In recent years, the knowledge on 14-3-3 functioning has grown rapidly. Multiple isoforms of 14-3-3 proteins are found in all tested eukaryotic organisms where they function, as homo- and heterodimers, by binding to distinct amino acid motifs in target proteins. Upon binding of a 14-3-3 protein, the functionality of the target protein is altered. This is accomplished by either direct activation/inactivation of the target, by (prevention of) translocation of the target to a cellular compartment or by facilitating interaction with other molecules. Further, the crystal structure of several 14-3-3 isoforms has been elucidated, what provided insight in the regions of the proteins that are involved in, for example, dimerisation and target interaction. Interestingly, these crystal structures of different 14-3-3 isoforms are essentially identical. This brings us to one of the current issues in 14-3-3 biology; are there functional differences between 14-3-3 isoforms? And if yes, what causes these differences and which isoform is responsible for the regulation of a certain target protein?

The amino acid sequence of the different 14-3-3 isoforms is very well conserved, especially the regions responsible for target binding. Still, several reports describe differences between 14-3-3 isoforms in their ability to act on target molecules. In line with these observations, clear differences between barley 14-3-3 isoforms in regulating the

slow-activating vacuolar (SV) channel were observed (Chapter 2). The barley SV current was reduced by 14-3-3B and 14-3-3C, but not by 14-3-3A. Further, we studied the ability of 14-3-3 isoforms to bind a distinct 14-3-3 binding motif found in the N-terminal region of KCO1, which was believed to be a component of the SV channel at that time. Surprisingly, 14-3-3A interacted with the highest affinity to a peptide representing this binding motif in barley KCO1, followed by the B- and C- isoform. This discrepancy can be explained by the current knowledge that the SV channel is formed by TPC1, and not by KCO1. The bright side of this is that these results now provide two additional examples of functional differences between 14-3-3 isoforms.

The apparent functional differences between 14-3-3 isoforms were further explored in Chapter 3 using one of the model systems for 14-3-3 action in plants, the inhibition of nitrate reductase (NR). Since some reports suggest that differential expression and availability of 14-3-3 isoforms, rather than their functional capability, determines which isoform regulates a certain target protein, both the availability and capability of the 14-3-3 isoforms to inhibit NR were analysed. We found that 14-3-3C is unavailable in dark harvested barley leaf extract and 14-3-3A is functionally not capable to efficiently inhibit NR activity, leaving 14-3-3B as the only characterized isoform able to regulate NR in barley. Further, using site directed mutagenesis, we identified a single amino acid variation (Gly versus Ser) in loop 8 of the 14-3-3 proteins that plays an important role in the observed functional specificity. Mutating the Gly residue of 14-3-3A to the alternative residue, as found in 14-3-3B and 14-3-3C, turned it into a potent inhibitor of NR activity.

These results show that both the availability of 14-3-3 isoforms as well as their ability to act on a target protein (isoform specificity) determine the biological function of a given isoform. For a good perspective of the role 14-3-3 proteins fullfil, it is therefore crucial to identify the different 14-3-3 targets and the availability and capability of 14-3-3 isoforms with respect to these targets. In Chapter 4 we describe the development of a small-scale affinity purification approach for the identification of 14-3-3 interacting proteins. Thus far, this approach led to the identification of NR, as a control, and neutral invertase as 14-3-3 interacting proteins. The novel 14-3-3 target, neutral invertase, is the next 14-3-3

target that serves a function in the carbohydrate metabolism (Chapter 1) and the activity of this enzyme is inhibited upon addition of 14-3-3C.

Further, in the light of the availability of 14-3-3 isoforms, two additional barley 14-3-3 isoforms (14-3-3D and 14-3-3E) were identified (Chapter 5) and the relative expression of the five different isoforms was determined in several barley tissues during different stages of development. The observed differences in expression provide an indication for the availability of 14-3-3 isoforms in regulating cellular processes. Finally, because of the possible involvement of 14-3-3 proteins in abscisic acid (ABA) signal transduction, the hormone that plays a key role during seed germination, special attention was paid to the expression of the 14-3-3 genes during the earliest hours of development and the results show an interesting increase in expression of some 14-3-3 isoforms in response to ABA.

In conclusion, barley contains at least five 14-3-3 isoforms which are differentially expressed throughout the plant. Observed functional isoform specificity between these 14-3-3 isoforms in the binding and regulating target proteins is, at least partially, the consequence of small variations in the 14-3-3 molecules that can be as little as a single amino acid residue. Differential availability in combination with functional isoform specificity determines the biological role for a given 14-3-3 isoform, as shown for the regulation of NR. It is therefore likely that each 14-3-3 isoform is responsible for the regulation of a subset of target proteins.

Samenvatting

Samenvatting

Cellulaire processen zoals de celcyclus, ion transport, gen expressie en enzym activiteit worden nauwgezet gereguleerd, bijvoorbeeld om het ontstaan van tumoren te voorkomen, homeostase te onderhouden en adequaat op veranderingen in de omgeving te reageren (zoals lichtintensiteit, osmotische potentiaal en de aanwezigheid van ziekteverwekkers). Het begrijpen van de mechanismen waarmee organismen/cellen de activiteit en functie van eiwitten reguleren, en daarmee van cellulaire processen, zou kunnen leiden tot het manipuleren van deze regulatiemechanismen om bijvoorbeeld ziekten/infecties te behandelen of gewasopbrengst te verhogen. Het werk dat in dit proefschrift wordt beschreven, heeft tot doel een beter inzicht te verkrijgen in de rol en functie van een specifieke klasse van regulatoren, de familie van 14-3-3 eiwitten.

Zoals beschreven in Hoofdstuk 1, is gebleken dat de familie van 14-3-3 eiwitten belangrijke regulatoren zijn van veel eiwitten die betrokken zijn bij een grote verscheidenheid aan cellulaire processen. De laatste jaren is de kennis rond het functioneren van 14-3-3 eiwitten snel toegenomen. Meerdere 14-3-3 isovormen zijn aangetoond in alle onderzochte eukaryote organismen, waar ze functioneren als homo- en heterodimeren door te binden aan een specifieke aminozuurvolgorde in bindingspartners. Door binding van een 14-3-3 eiwit verandert de functionaliteit van de bindingspartner. Dit wordt verwezenlijkt door directe activering/inactivering van de bindingspartner, door (preventie van) translocatie van de bindingspartner naar een ander cellulair compartiment of door een interactie met andere moleculen te faciliteren. Verder is de kristalstructuur van verschillende 14-3-3 isovormen opgehelderd, hetgeen inzicht verschaft in welke gedeelten van de eiwitten betrokken zijn bij bijvoorbeeld dimerisatie en interactie met de bindingspartner. De kristalstructuren van deze verschillende 14-3-3 isovormen zijn praktisch identiek, hetgeen ons brengt bij één van de huidige onderwerpen in 14-3-3 biologie; zijn de 14-3-3 isovormen functioneel verschillend? En zo ja, wat veroorzaakt deze verschillen en welke isovorm is verantwoordelijk voor de regulatie van een bepaalde bindingspartner?

De aminozuurvolgorde van de verschillende 14-3-3 isovormen is zeer goed geconserveerd, met name in die gedeelten die betrokken zijn bij de interactie met bindingspartners. Toch beschrijven meerdere publicaties verschillen tussen 14-3-3 isovomen in hun werking op bindingspartners. Overeenkomstig met deze observeringen werden duidelijke verschillen waargenomen tussen gerst 14-3-3 isovormen in de regulatie van het langzaam-activerende vacuolaire (SV) kaliumkanaal (Hoofdstuk 2). De stroom van het gerst SV-kanaal werd geremd door 14-3-3B en 14-3-3C, maar niet door 14-3-3A. Hiernaast bestudeerden we de mogelijkheden van de 14-3-3 isovormen om te binden aan de specifieke aminozuurvolgorde die aanwezig is in het N-terminale gedeelte van KCO1, een eiwit dat op dat moment verondersteld werd deel uit te maken van het SV-kanaal. Tot onze verrassing bleek 14-3-3A met de hoogste affiniteit te binden aan een peptide dat de specifieke amonizuurvolgorde van gerst KCO1 representeerde, op afstand gevolgd door 14-3-3B en 14-3-3C. Deze discrepantie kan worden verklaard door de huidige kennis dat het SV-kanaal wordt gevormd door TPC1, en niet door KCO1. Deze resultaten beschrijven nu dus twee extra voorbeelden van functionele verschillen tussen 14-3-3 isovormen.

De duidelijke functionele verschillen tussen 14-3-3 isovormen werden verder onderzocht in Hoofdstuk 3 met behulp van één van de modelsystemen voor 14-3-3 regulatie in planten, de remming van nitraatreductase (NR). Aangezien sommige wetenschappers suggereren dat differentiële expressie en beschikbaarheid van 14-3-3 isovormen, in plaats van hun functionele vermogen, bepalen welke isovorm een bepaalde bindingspartner reguleert, werden zowel de beschikbaarheid als het vermogen van 14-3-3 isovormen om NR te reguleren geanalyseerd. Wij vonden dat 14-3-3C niet aanwezig is in het, in het donker geoogste, extract van gerstblad en 14-3-3A functioneel niet in staat is om de activiteit van NR efficiënt te remmen. Hierdoor bleef 14-3-3B als enige van de beschreven isovormen over als regulator van NR in gerst. Verder hebben we, met behulp van mutagenese, een natuurlijk variërend aminozuur (Gly versus Ser) geïdentificeerd in loop 8 van de 14-3-3 eiwitten dat een belangrijke rol speelt in de waargenomen isovormspecificiteit. Mutatie van de glycine van 14-3-3A naar een serine, zoals aanwezig in 14-3-3B en 14-3-3C, veranderde 14-3-3A in een efficiënte remmer van NR activiteit.

Deze resultaten laten zien dat zowel de beschikbaarheid van 14-3-3 eiwitten als het vermogen om interactie aan te gaan met een bindingspartner (isovormspecificiteit) de biologische functie van een bepaalde isovorm bepalen. Om een beter inzicht te verkrijgen in de rol die 14-3-3 eiwitten spelen is het daarom cruciaal om de verschillende bindingspartners te identificeren en de beschikbaarheid en het functionele vermogen van 14-3-3 eiwitten met betrekking tot deze bindingspartners te bepalen. In Hoofdstuk 4 beschrijven we de ontwikkeling van een affiniteit-opzuiveringsmethode om op kleine schaal 14-3-3 bindingspartners te identificeren. Tot nu toe heeft deze methode geresulteerd in de identificatie van NR, als controle, en de neutrale invertase als 14-3-3 bindingspartners. Het nieuwe 14-3-3 bindende eiwit, de neutrale invertase, is wederom een 14-3-3 bindingspartner die een rol heeft in de koolhydraat stofwisseling (Hoofdstuk 1) en de activiteit van dit enzym wordt geremd door 14-3-3C.

Tevens hebben we, in het kader van de beschikbaarheid van 14-3-3 isovormen, twee nieuwe 14-3-3 isovormen (14-3-3D en 14-3-3E) in gerst geïdentificeerd (Hoofdstuk 5) en de relatieve expressie van de vijf isovormen zijn onderzocht in verschillende gerstweefsels gedurende meerdere ontwikkelingsfases. De geobserveerde verschillen in expressie geven een indicatie voor de beschikbaarheid van 14-3-3 eiwitten in het reguleren van cellulaire processen. Vanwege de mogelijke rol die 14-3-3 eiwitten spelen in abscinezuur (ABA) signaaltransductie, het hormoon dat een sleutelrol vervult gedurende zaadkieming, is er extra aandacht besteed aan de expressie van de 14-3-3 genen in de eerste uren van de ontwikkeling. De resultaten laten een interessante toename van de expressie zien na toediening van ABA.

Samenvattend, gerst bevat minstens vijf 14-3-3 isovormen die verschillend tot expressie komen in de plant. De geobserveerde functionele isovormspecificiteit tussen deze 14-3-3 eiwitten in het binden en reguleren van bindingspartners zijn, op zijn minst gedeeltelijk, het gevolg van kleine variaties in de 14-3-3 eiwitten die beperkt kunnen zijn tot één enkel aminozuurresidu. Verschillen in beschikbaarheid in combinatie met functionele isovorm-specificiteit bepalen de biologische rol van een 14-3-3 isovorm, zoals aangetoond voor de regulatie van NR. Het is daarom waarschijnlijk dat elke 14-3-3 isovorm verantwoordelijk is voor de regulatie van een deel van de bindingspartners.

List of publications

List of publications

Sinnige, M.P., Roobeek, I., Bunney, T.D., Visser, A.J.W.G., Mol, J.N.M., De Boer, A.H. (2006) Single amino acid variation in barley 14-3-3 proteins leads to functional isoform specificity in het regulation of nitrate reductase. The Plant Journal, **44** (6), 1001-1009

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Submitted manuscripts

Sinnige, M.P. and De Boer, A.H. Regulation and discrimination by 14-3-3 proteins.

Schoonheim, P.J., **Sinnige, M.P.**, Casaretto, J.A., Bunney, T.D., Quatrano, R.S., De Boer, A.H. 14-3-3 adapter proteins are intermediates in ABA signal transduction during seed germination.

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Dankwoord

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